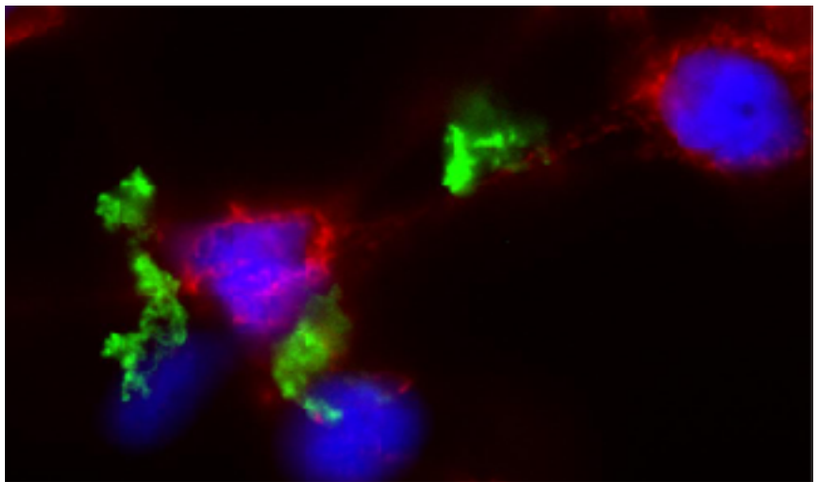
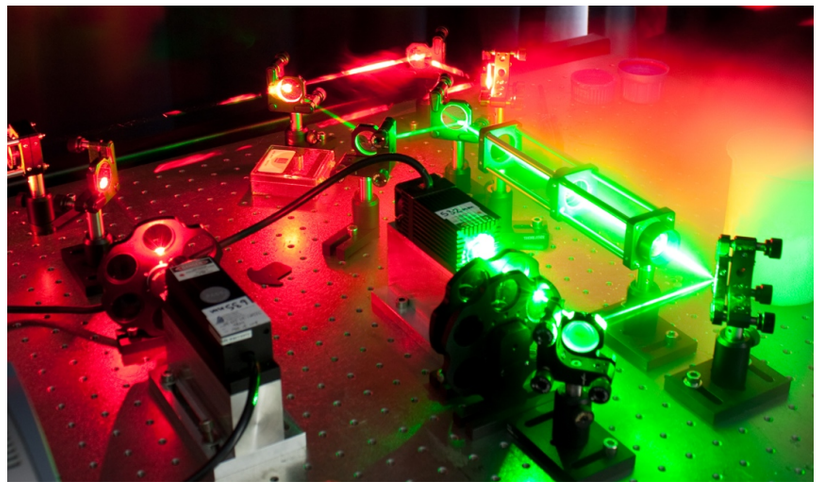
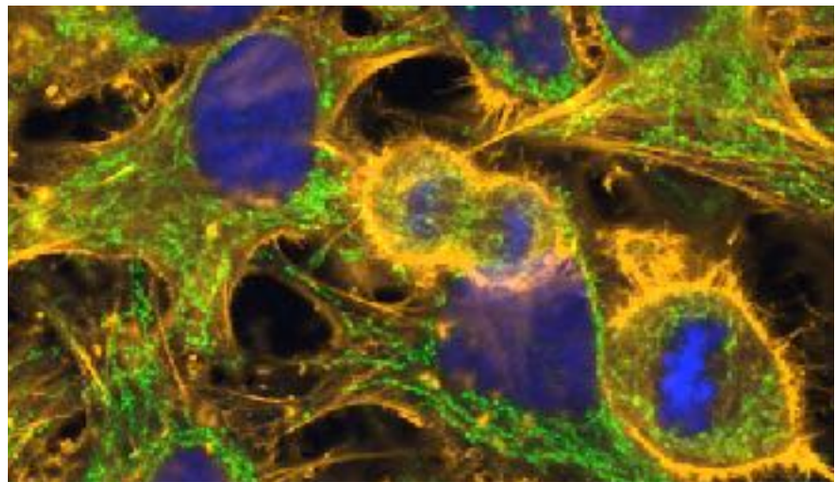
Understanding and Applying Fluorescence Microscopy







Carina Mónico

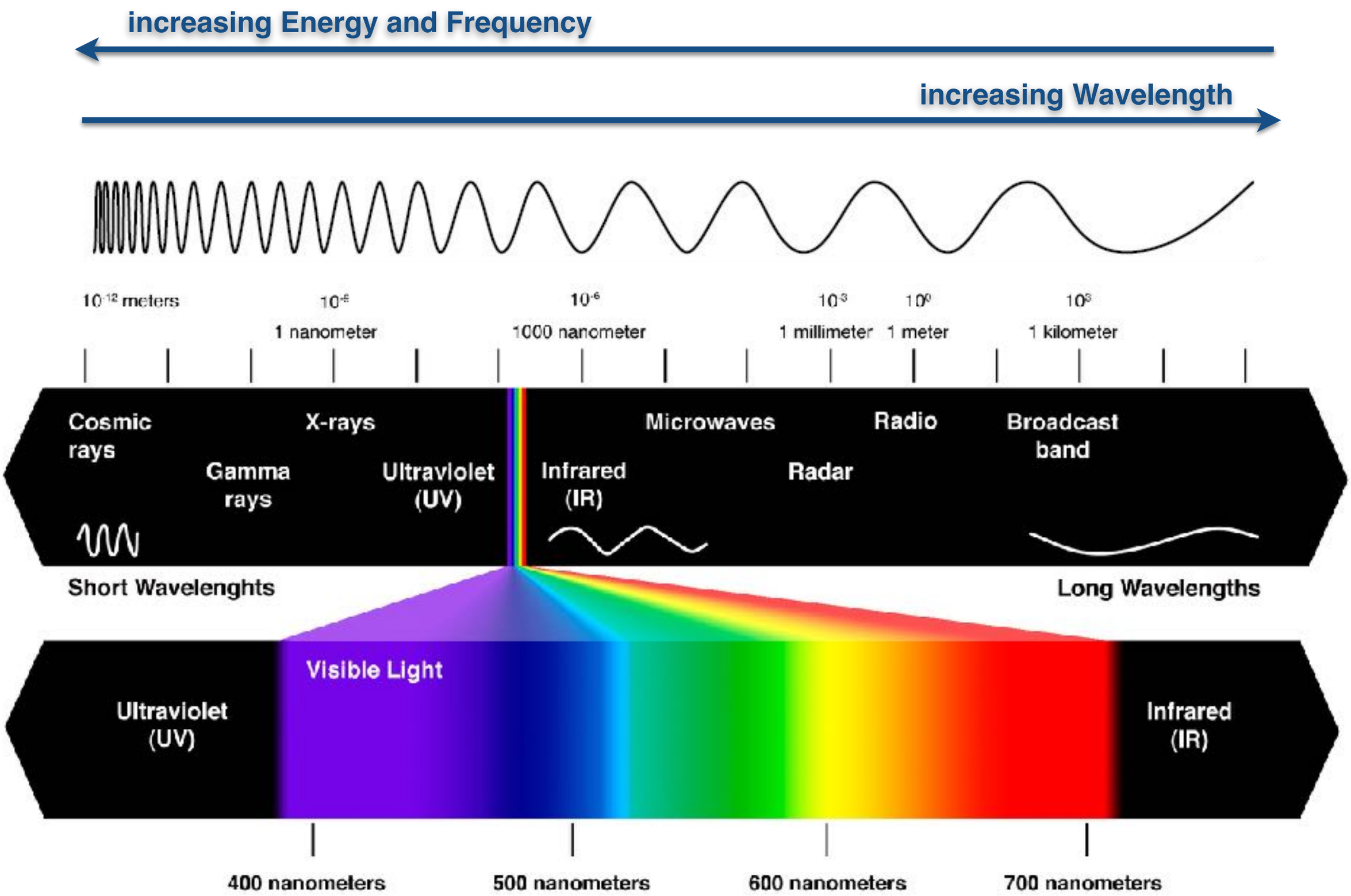
Micron assistant manager

Department of Biochemistry

Outline

- What is fluorescence?
- Why fluorescence?
- Principle and components of the fluorescence microscope
- Fluorescent light sources
- Fixation for light microscopy
- PSFs and OTFs

Light: the electromagnetic spectrum

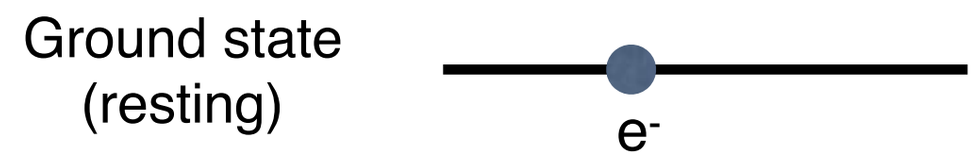


380 – 700 nm visible to the human eye

What is Fluorescence?

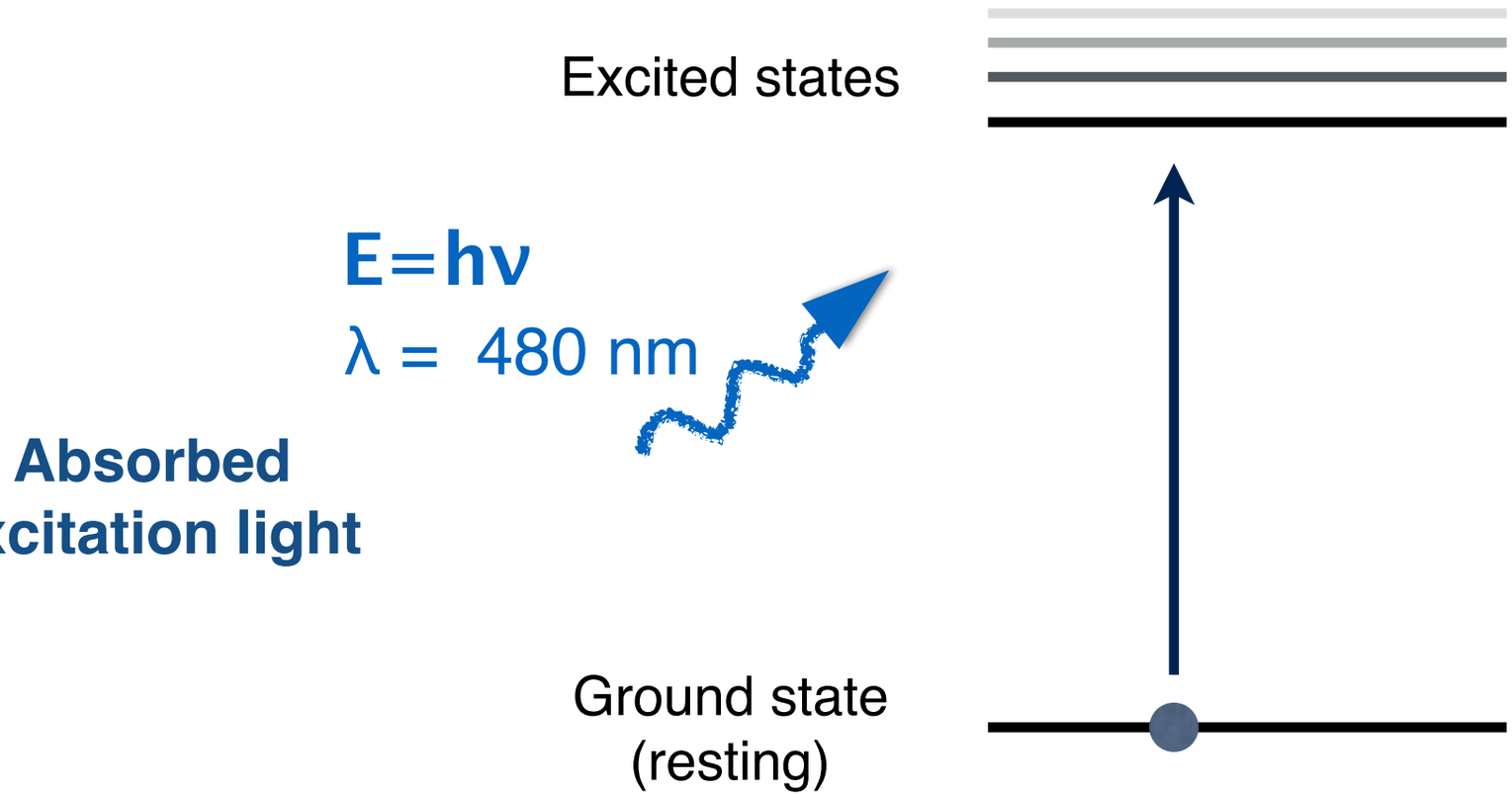
"**Fluorescence** is the emission of light by a substance that has absorbed light"

https://en.wikipedia.org/wiki/Fluorescence



Molecules have discrete levels of energy

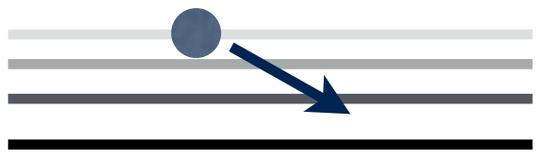
What is Fluorescence?



A photon is the energy unit for light to interact with matter

What is Fluorescence?

Excited states



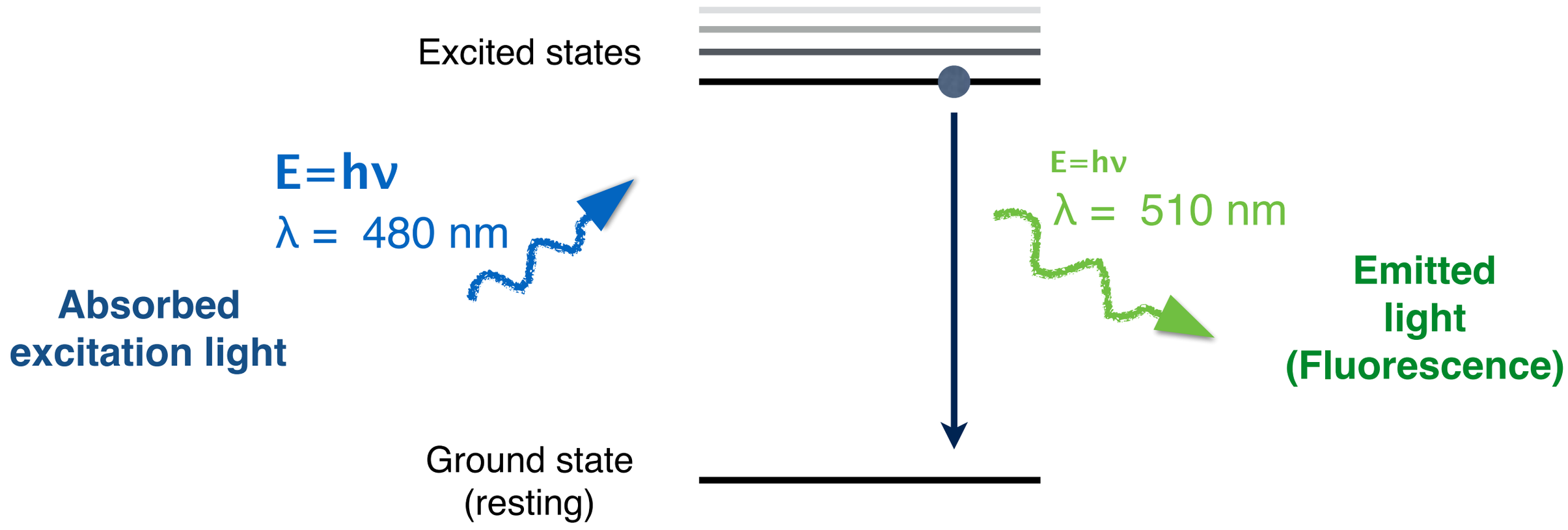
E=hν

λ = 480 nm

Absorbed excitation light

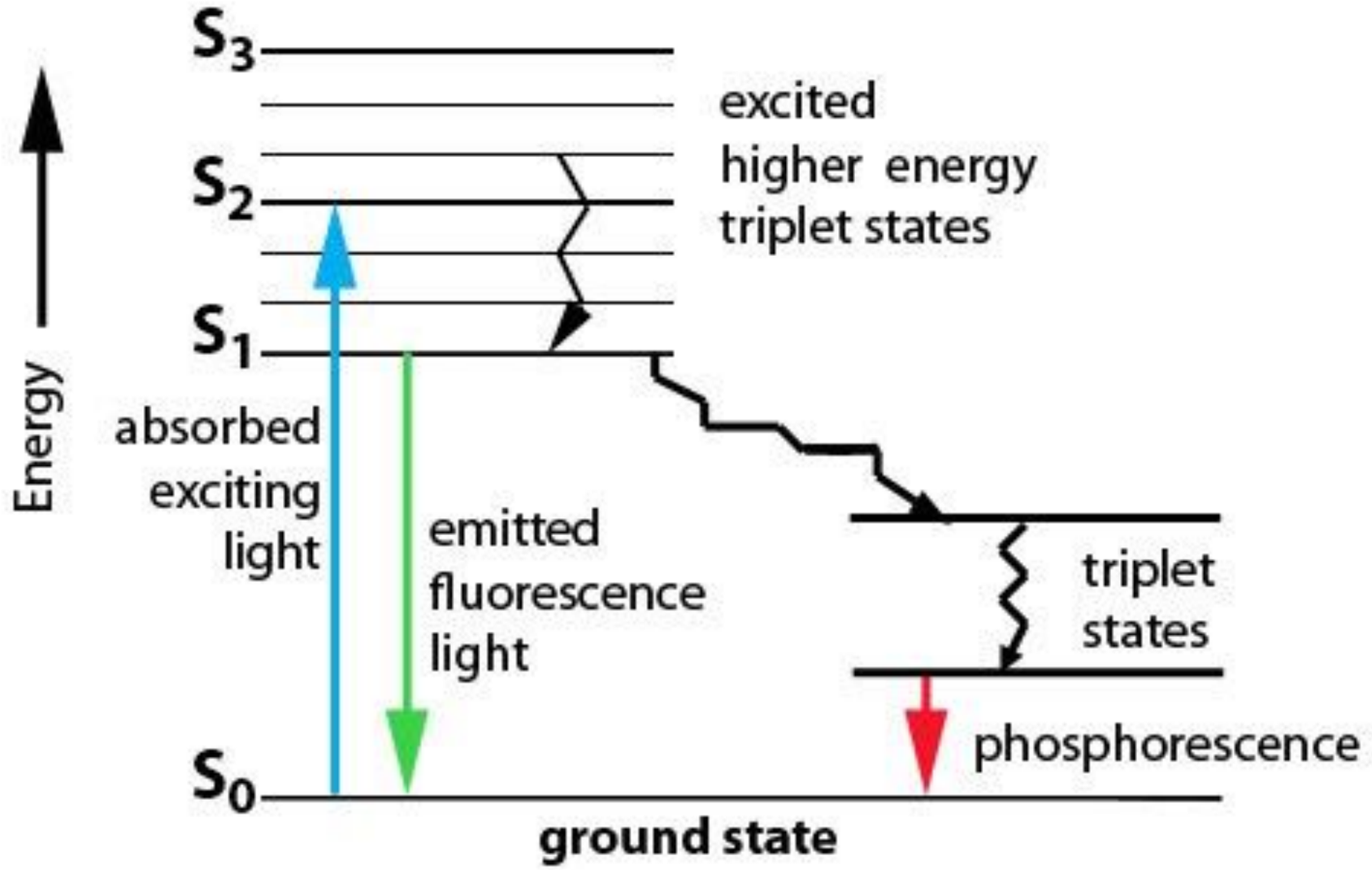
Ground state (resting)

What is Fluorescence?



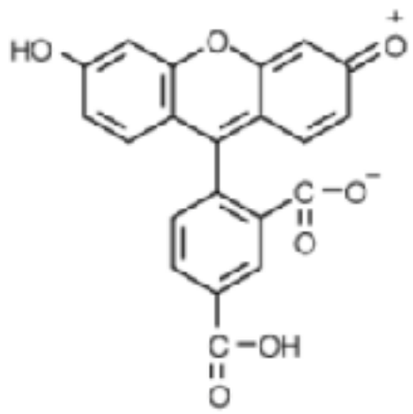
The full picture is represented on the Jablonski diagram…

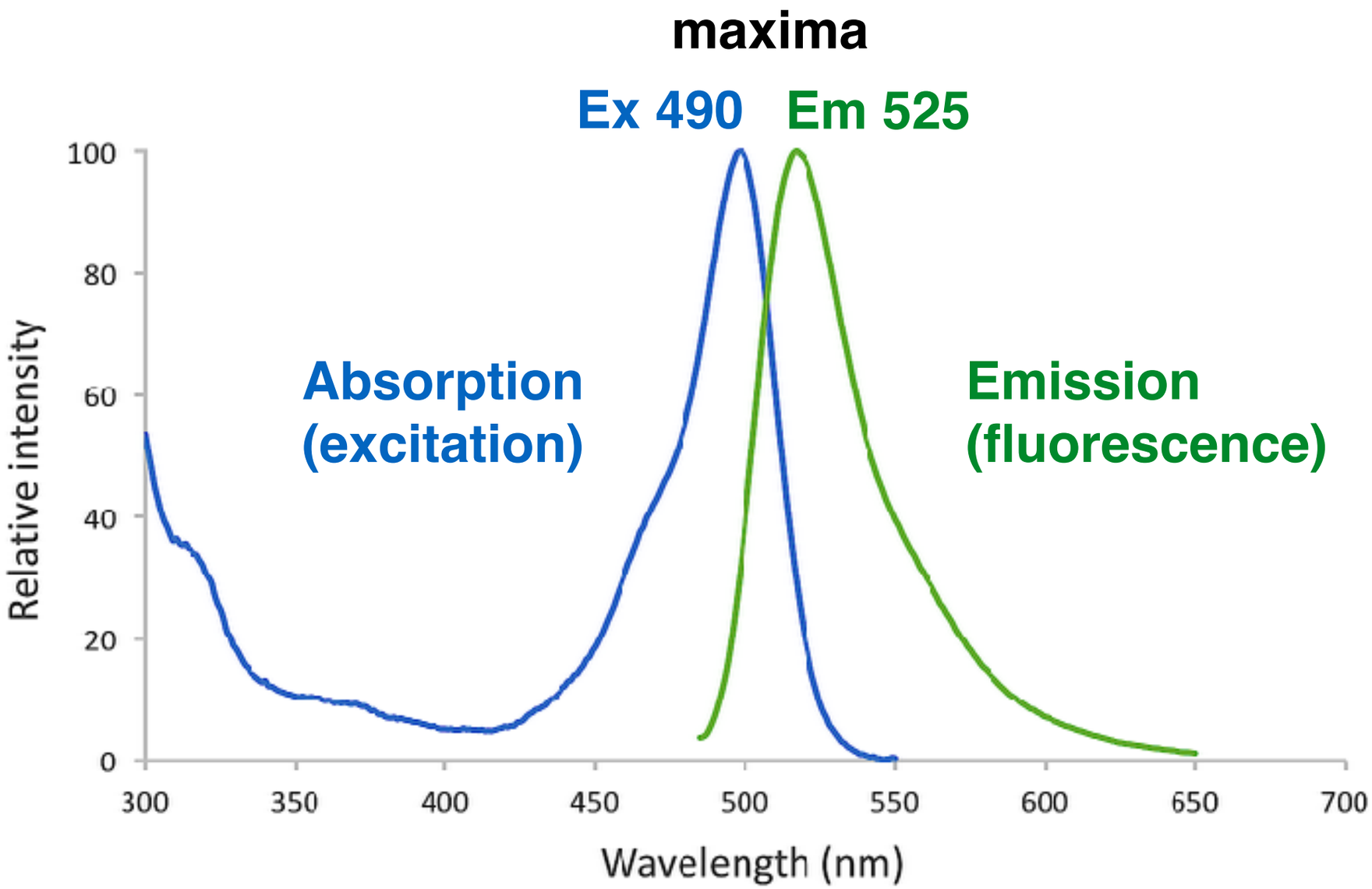
→ Lecture 5



Fluorescence Spectra

Fluorescein (FITC)





Fluorescent probes for microscopy

→ Lecture 5

Genetically encoded fluorescent proteins

- GFP, YFP, mCherry

Organic dyes

- Alexa, ATTO, Fluorescein, DAPI
- Fluorescent labelled antibodies (immunofluorescence)

Inorganic dyes

- Quantum Dots

Endogenous species

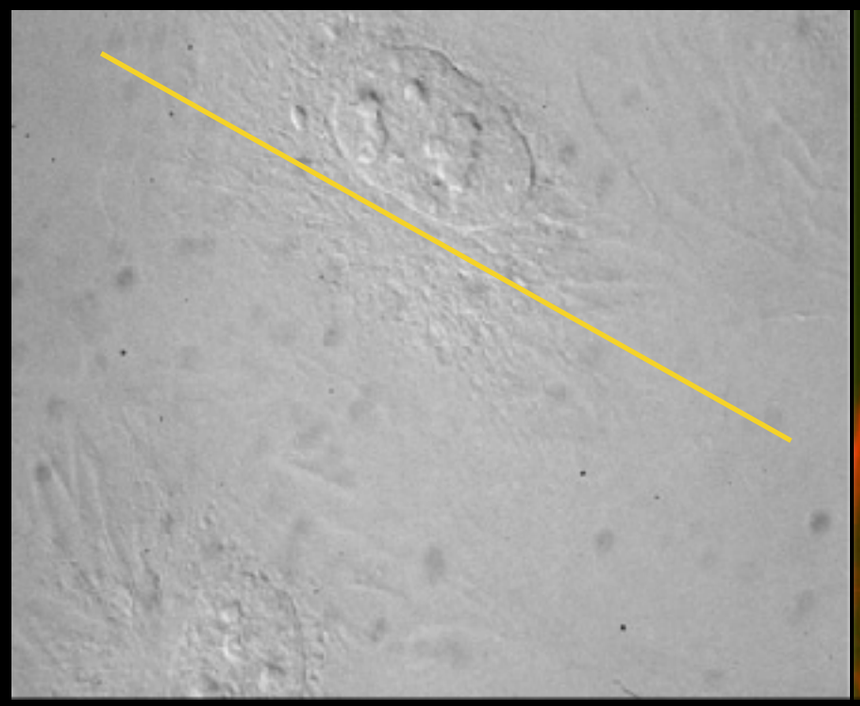
- Elastin, collagen, metabolic coenzymes (NADH, FAD)

Why Fluorescence?

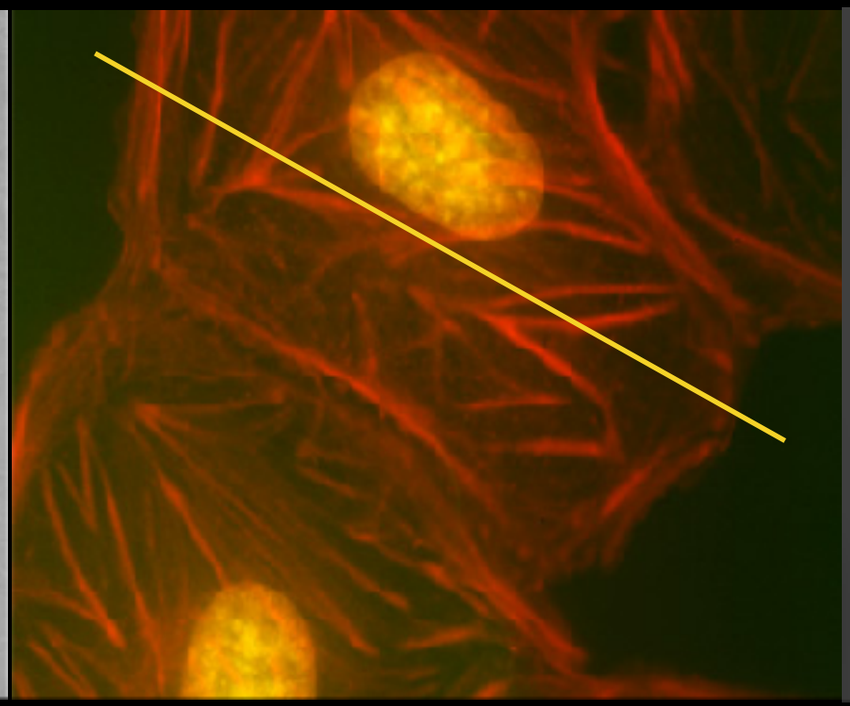
Chris Teren: https://www.youtube.com/watch?v=PhclTQ3g0s8

CONTRAST

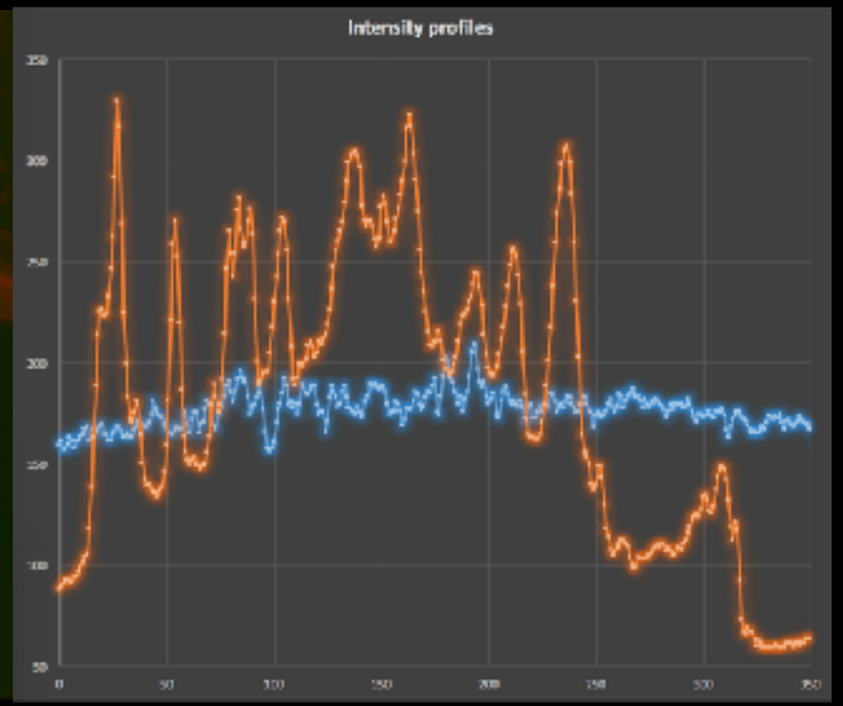
Why Fluorescence?



bright field (DIC)



fluorescence



Intensity profile

- Weak signal against dark background is easier to measure
- High signal to background - contrast

Why Fluorescence?

- Selective labeling
- Ease of multiplexing
- Quantitative

Dr Alexis J. Lomakin www.olympusbioscapes.com/gallery/2011/hm29.html

How do we get a black background….?

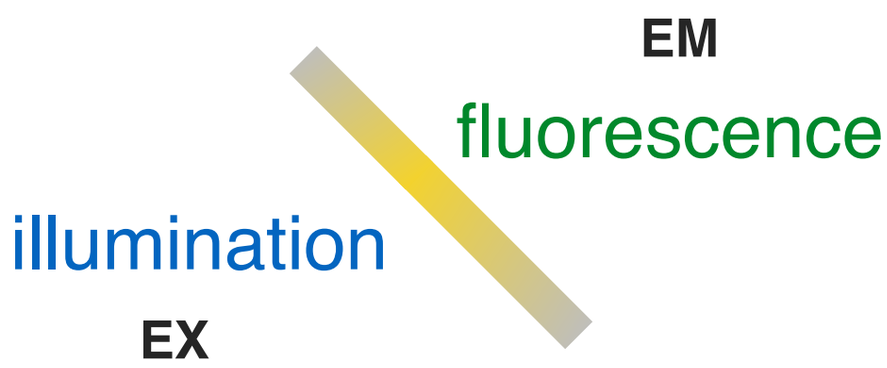
Fundamental problem in fluorescence microscopy

STRONG illumination

WEAK fluorescence signal

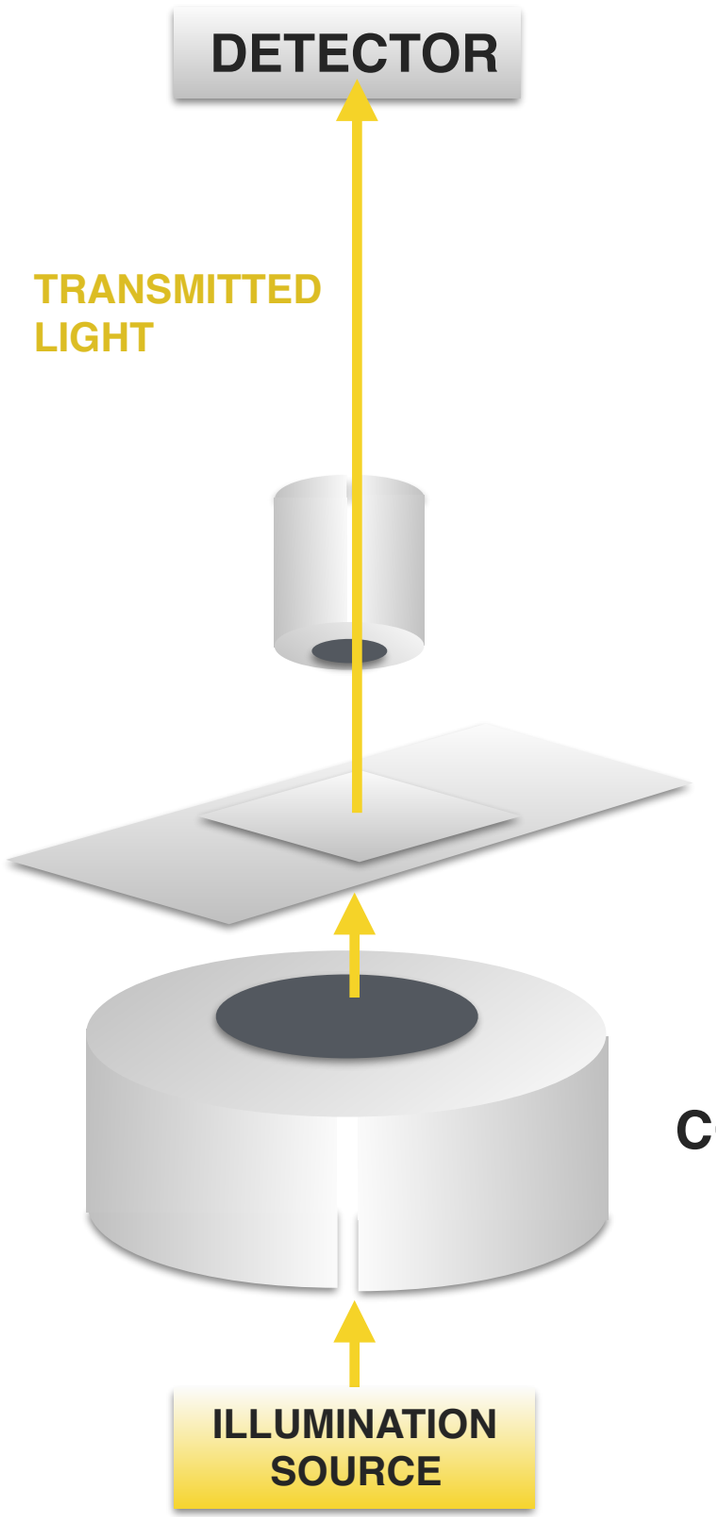
produce high-efficiency illumination of the specimen

while simultaneously capturing weak fluorescence emission

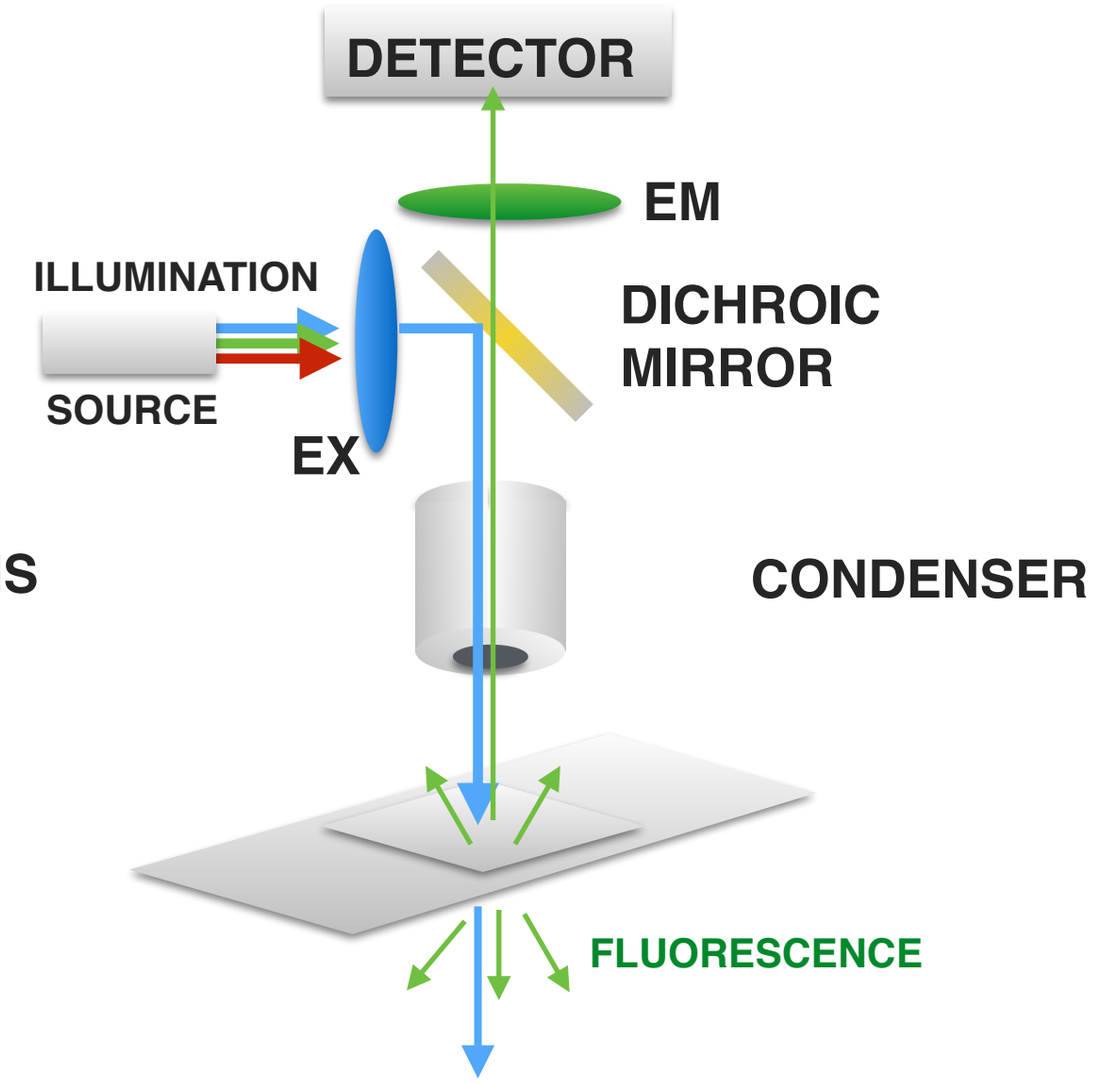


Components of a fluorescence microscope

Transmitted light (Brightfield)

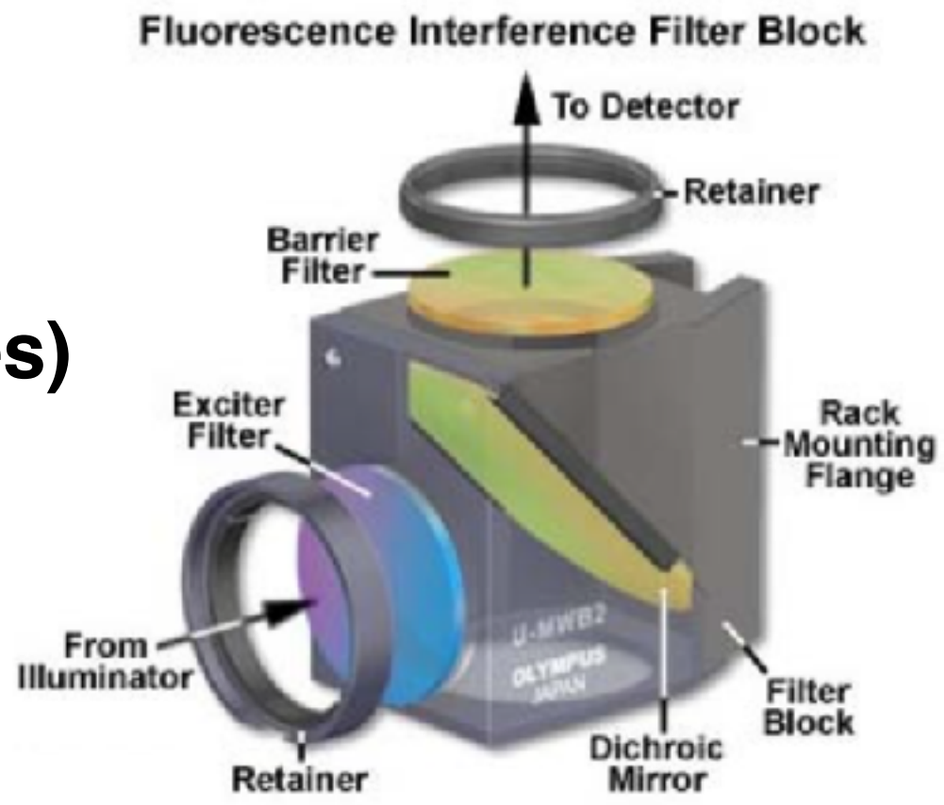


Reflected Light (Epifluorescence)



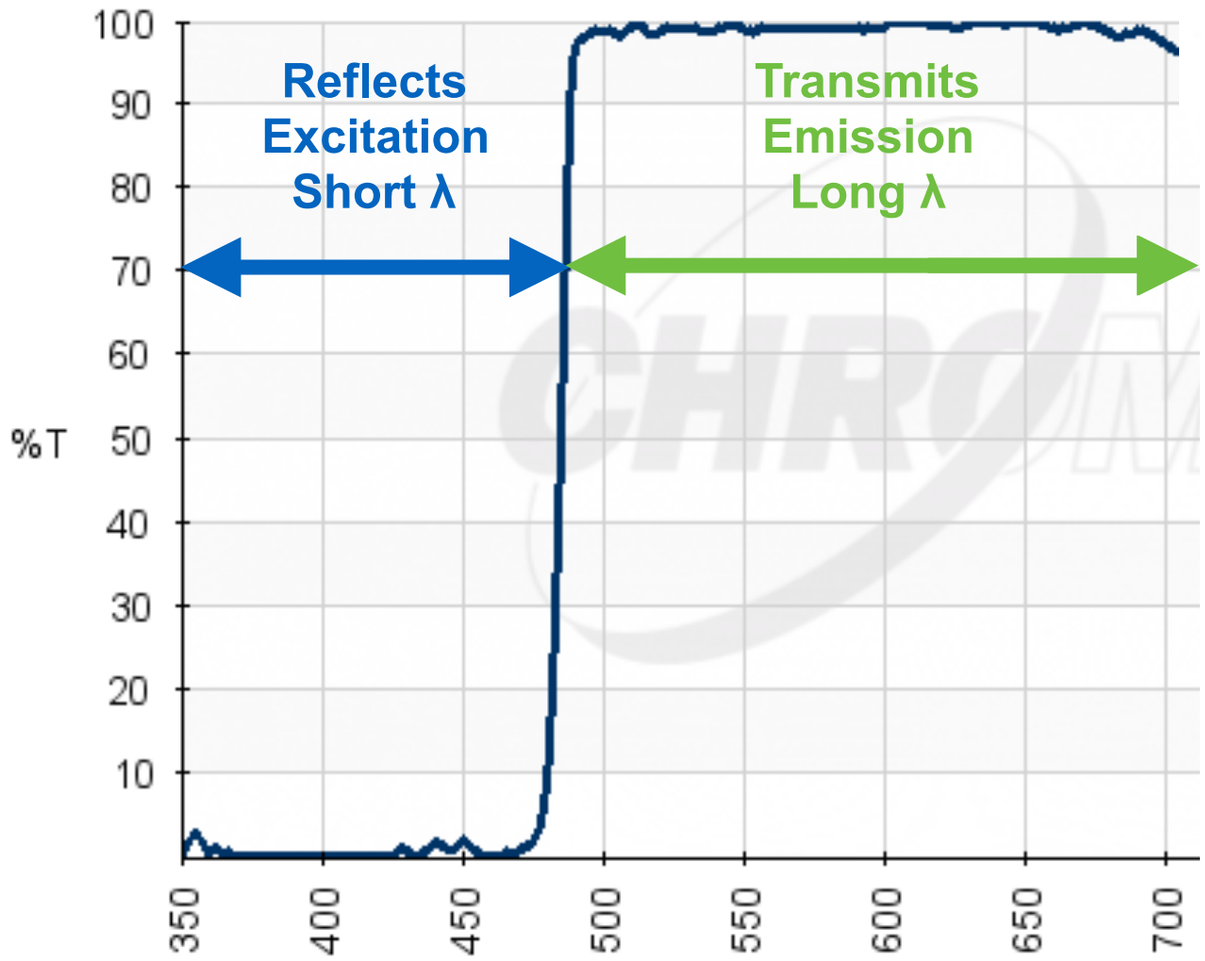
Epifluorescence vs Transillumination light paths (inverted)

Dichroic beamsplitter - at the heart of fluorescence microscopy

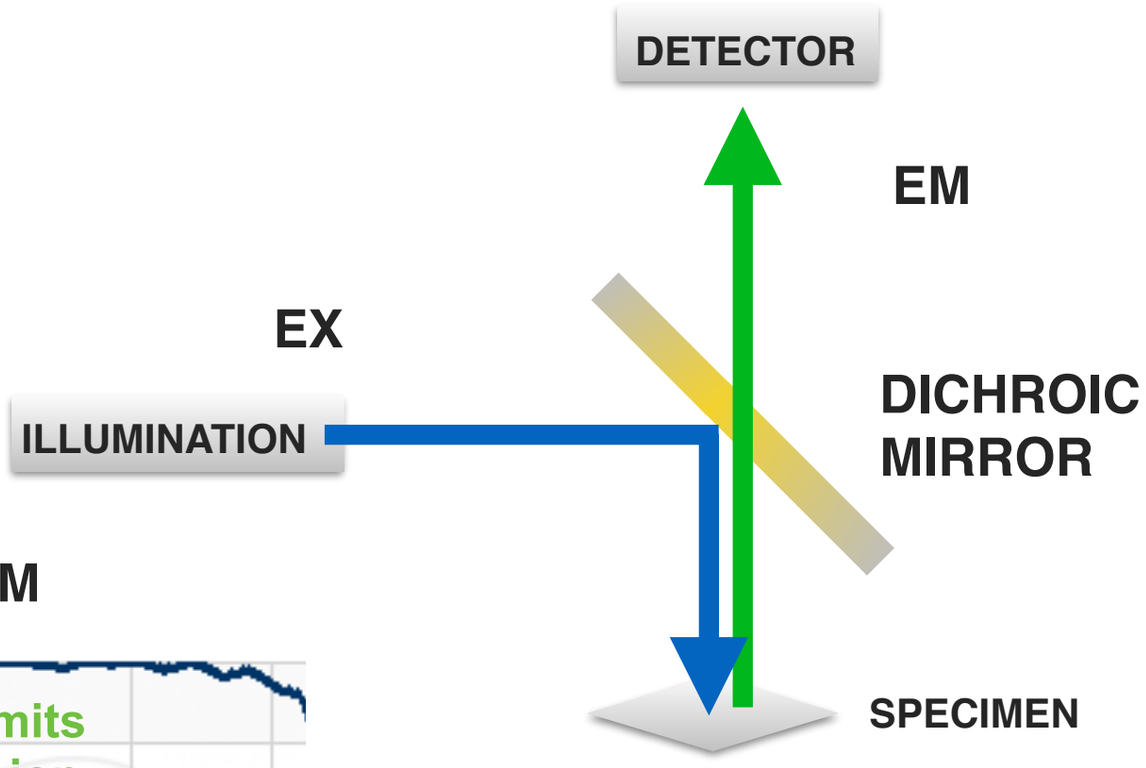


typical Dichroic (spectral properties)

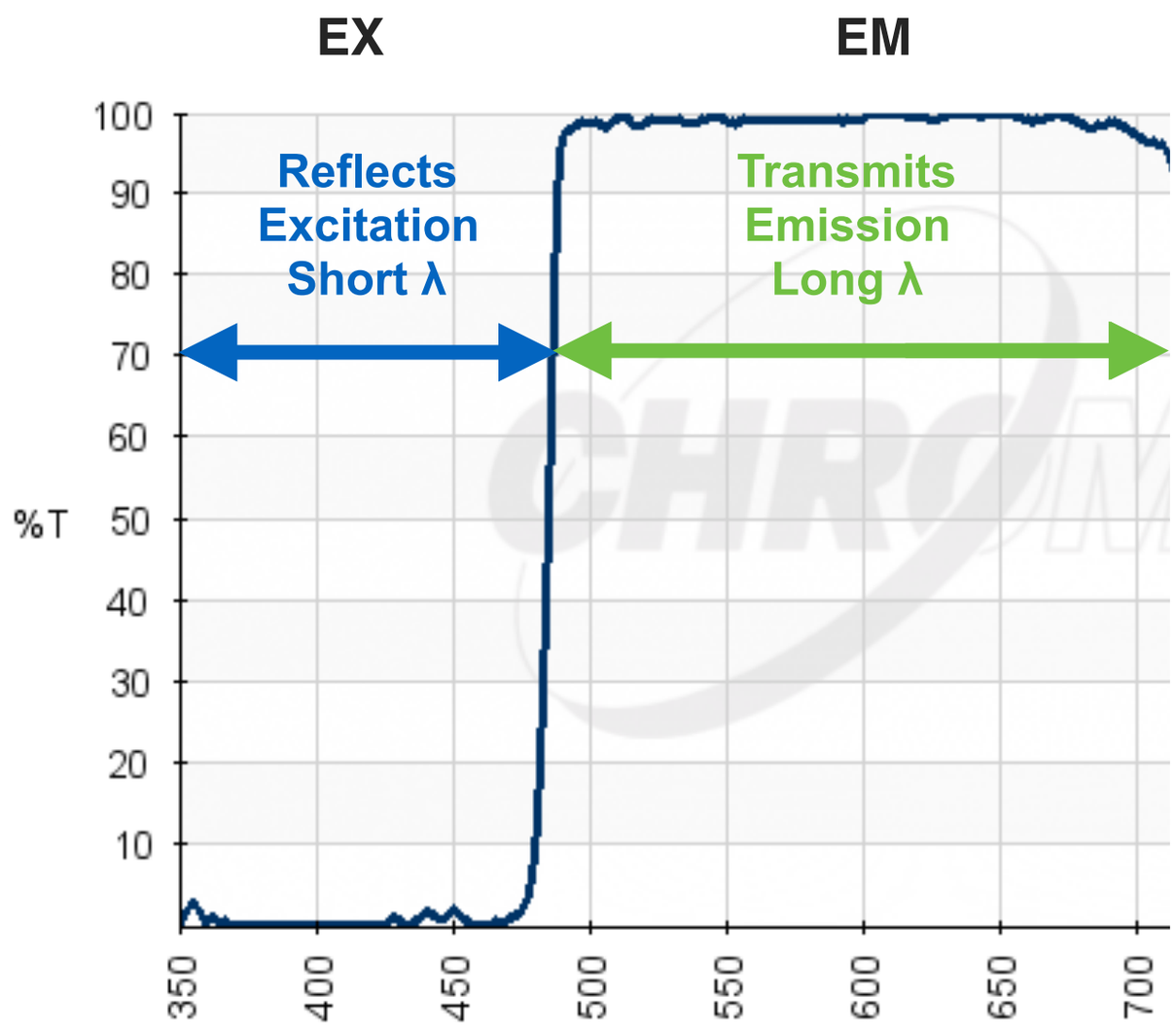
100% T = 0% R



Dichroic beamsplitter - at the heart of fluorescence microscopy



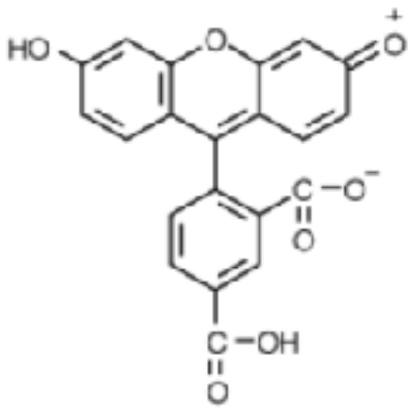
100% T = 0% R

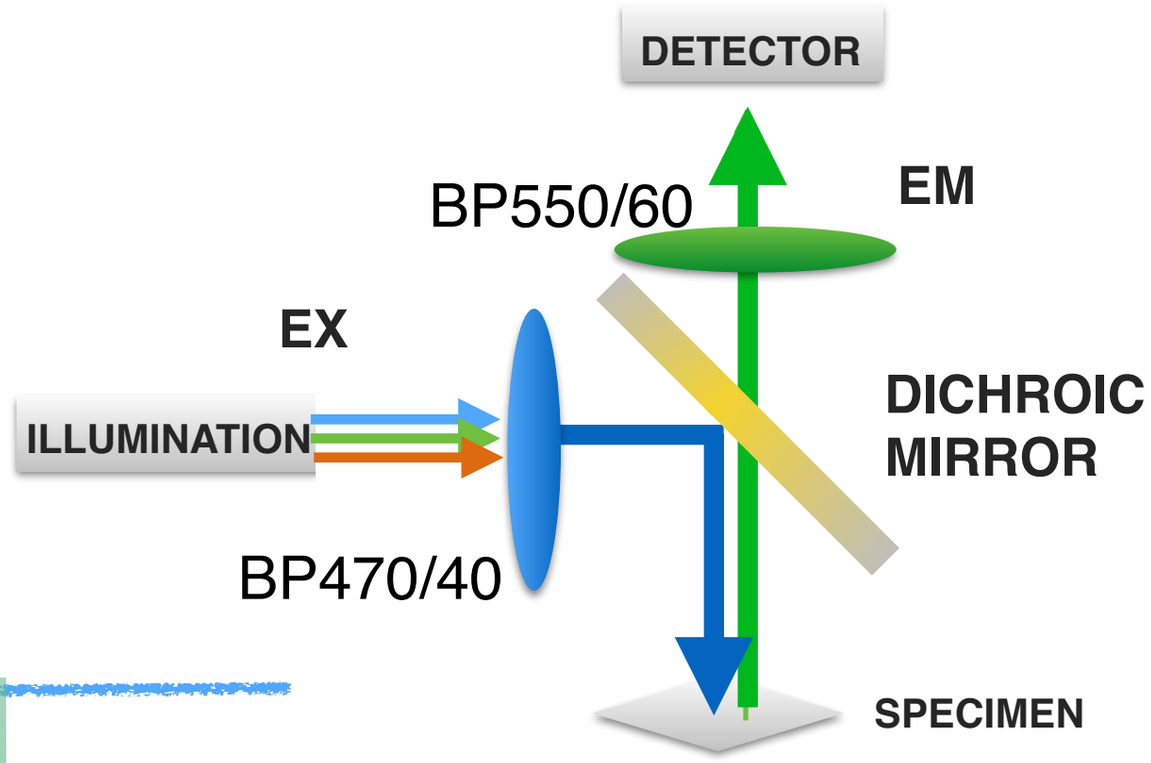


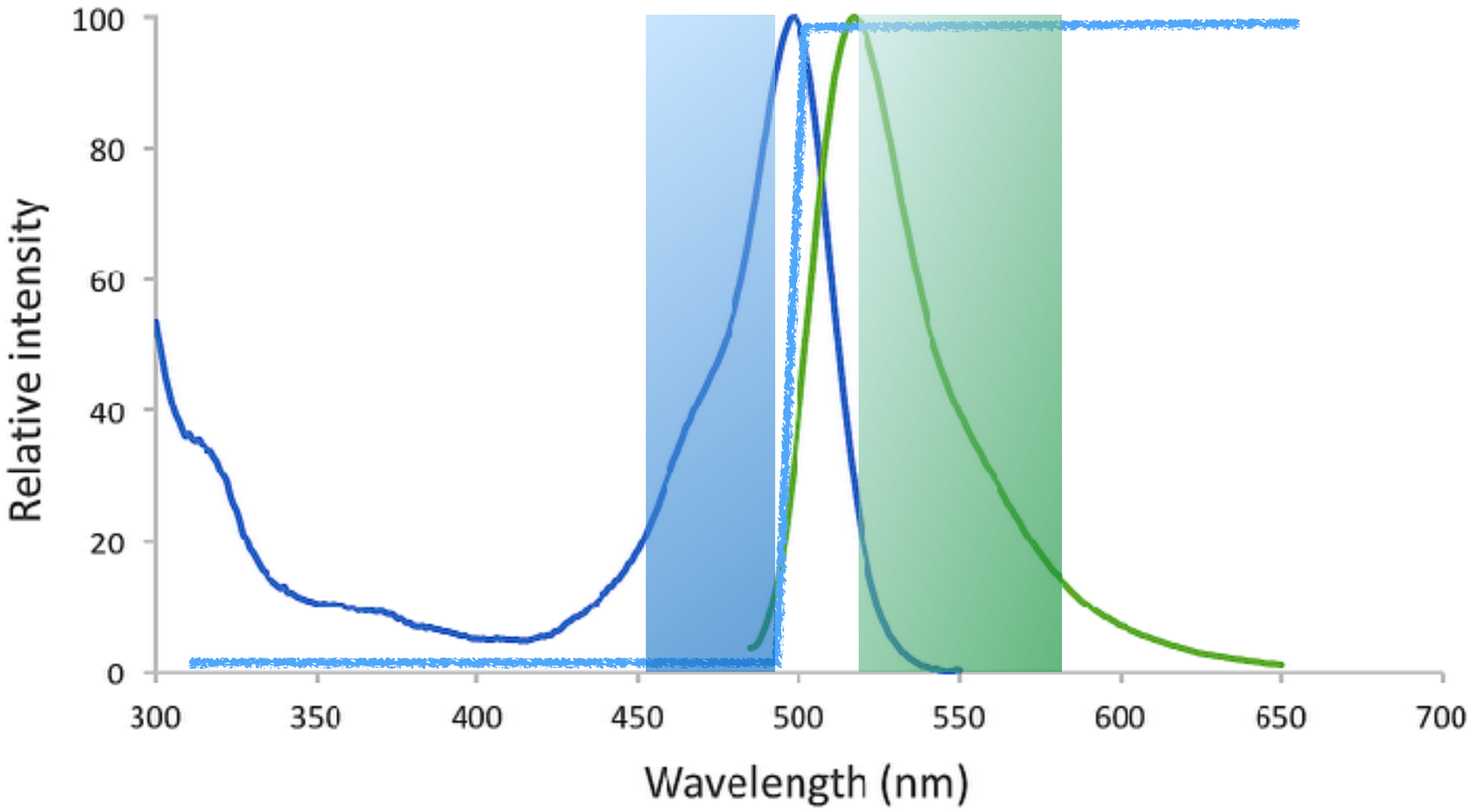
Dichroic beamsplitter - at the heart of fluorescence microscopy

… relate to dye spectrum

Fluorescein (FITC)

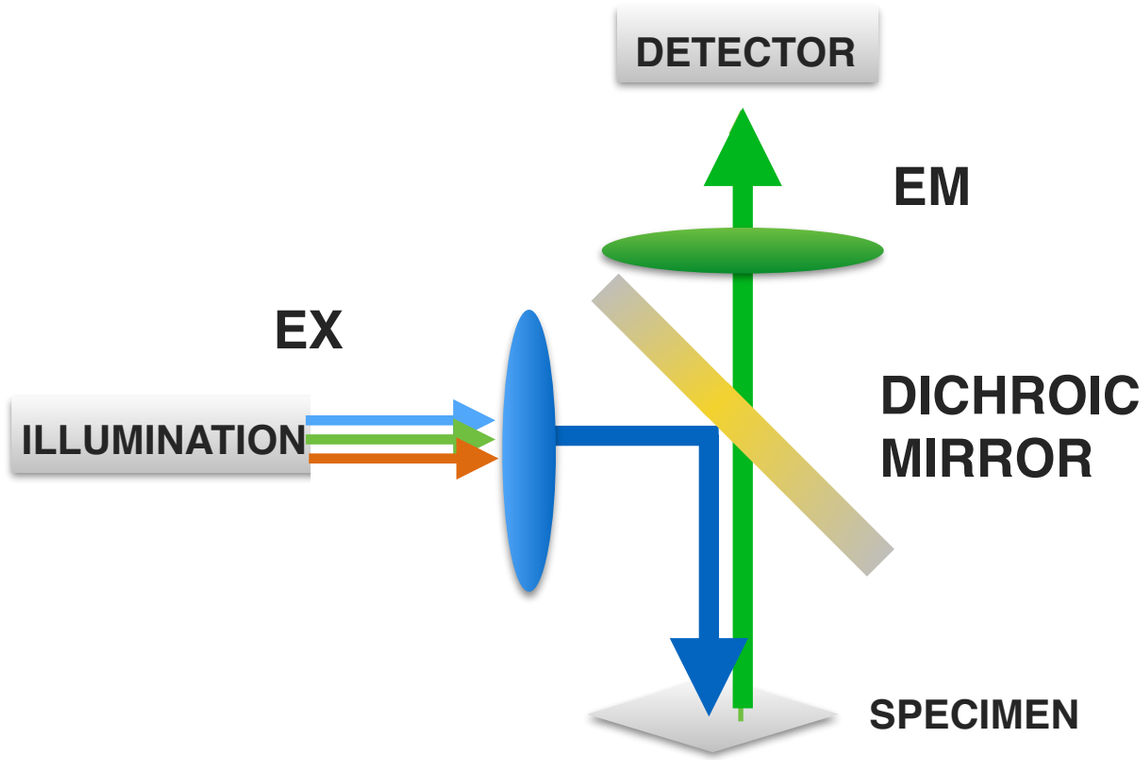






Dichroic beamsplitter - at the heart of fluorescence microscopy

… what about multiplexing…?



http://www.olympusmicro.com/

Dichroic beamsplitter - at the heart of fluorescence microscopy

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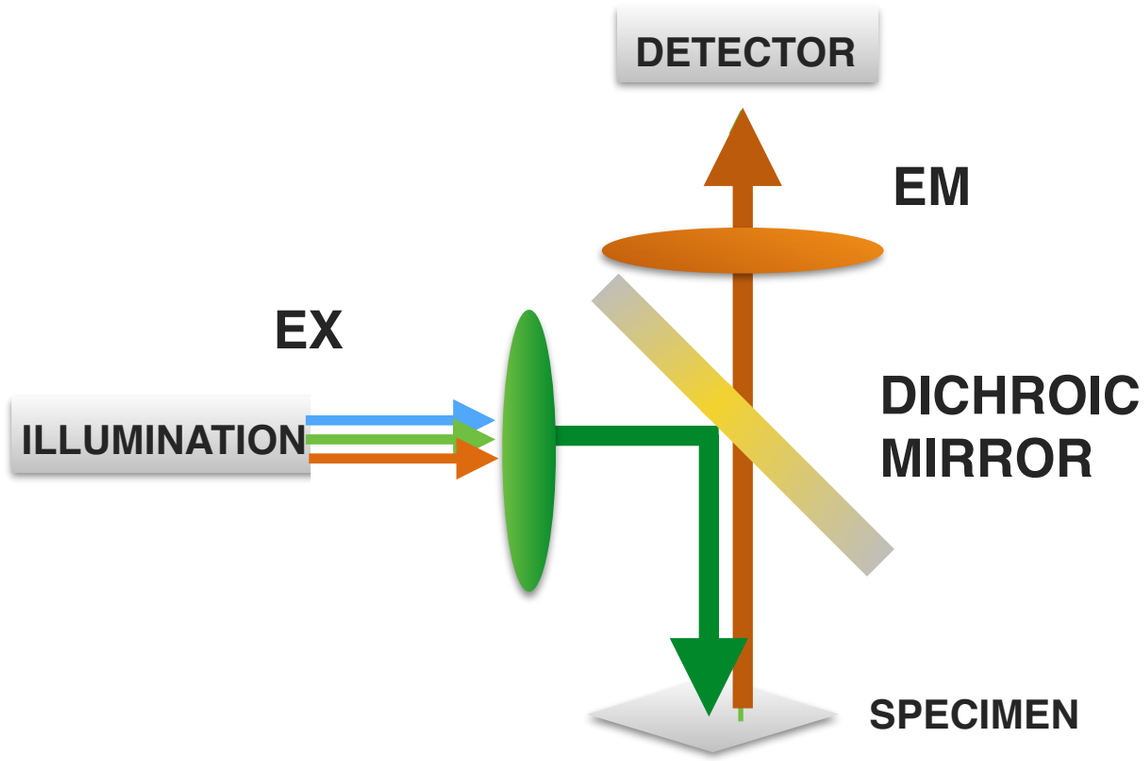

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Dichroic beamsplitter - at the heart of fluorescence microscopy

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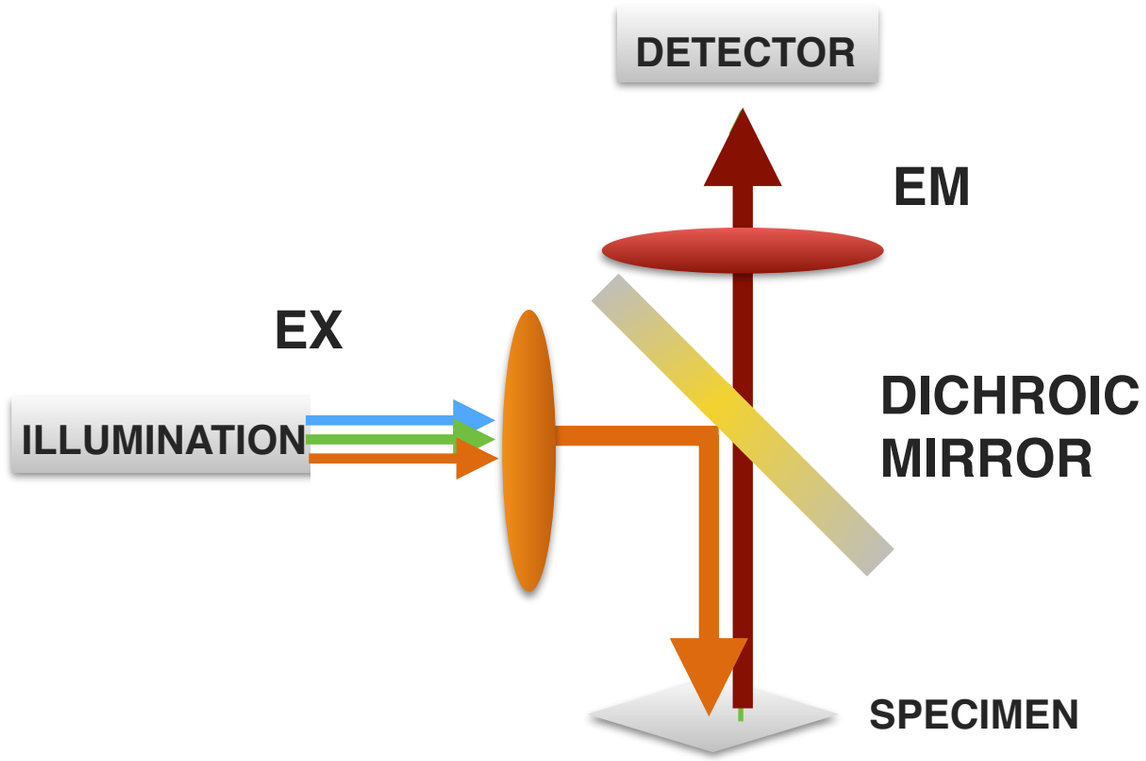

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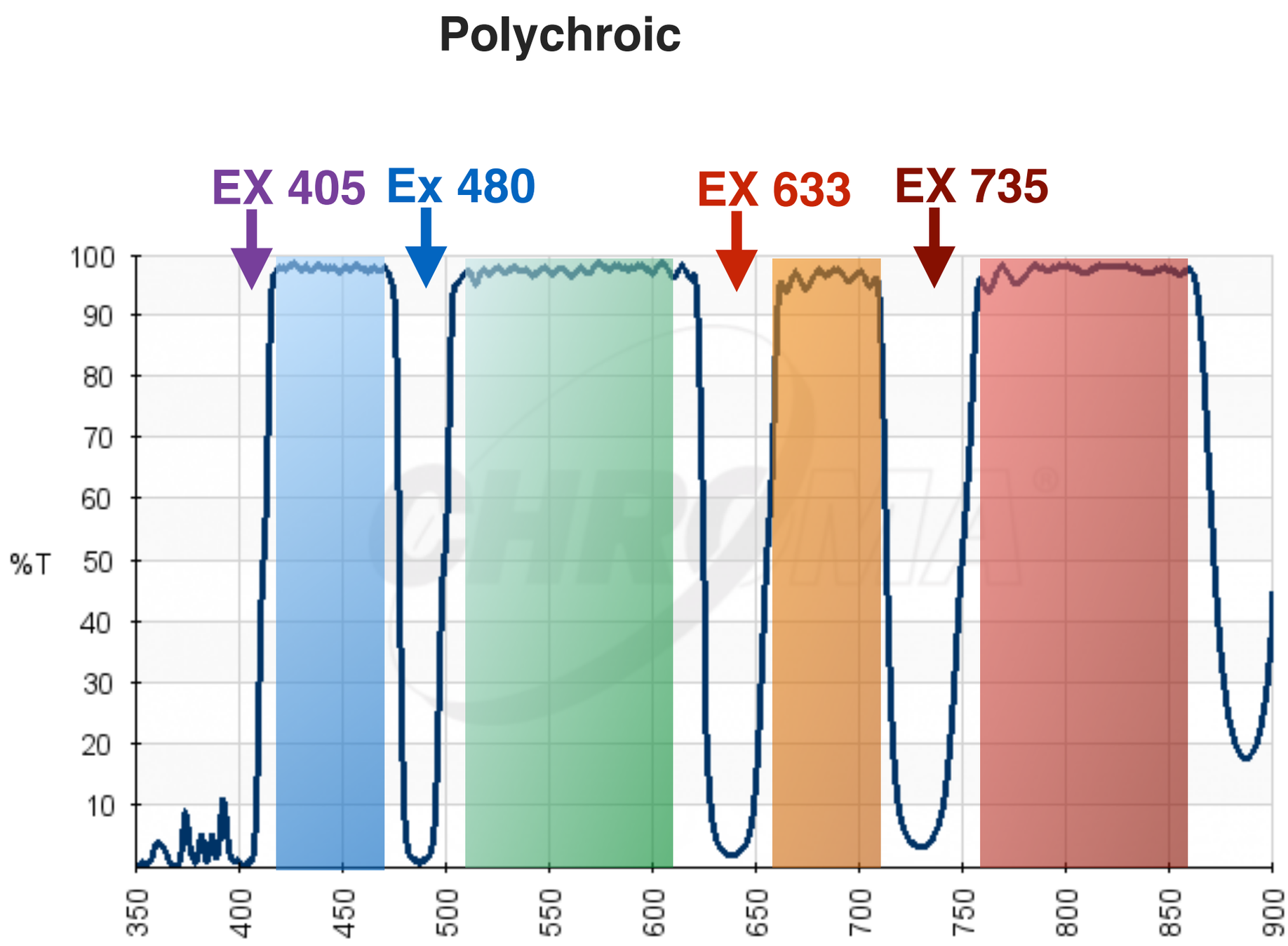
Dichroic beamsplitter - at the heart of fluorescence microscopy

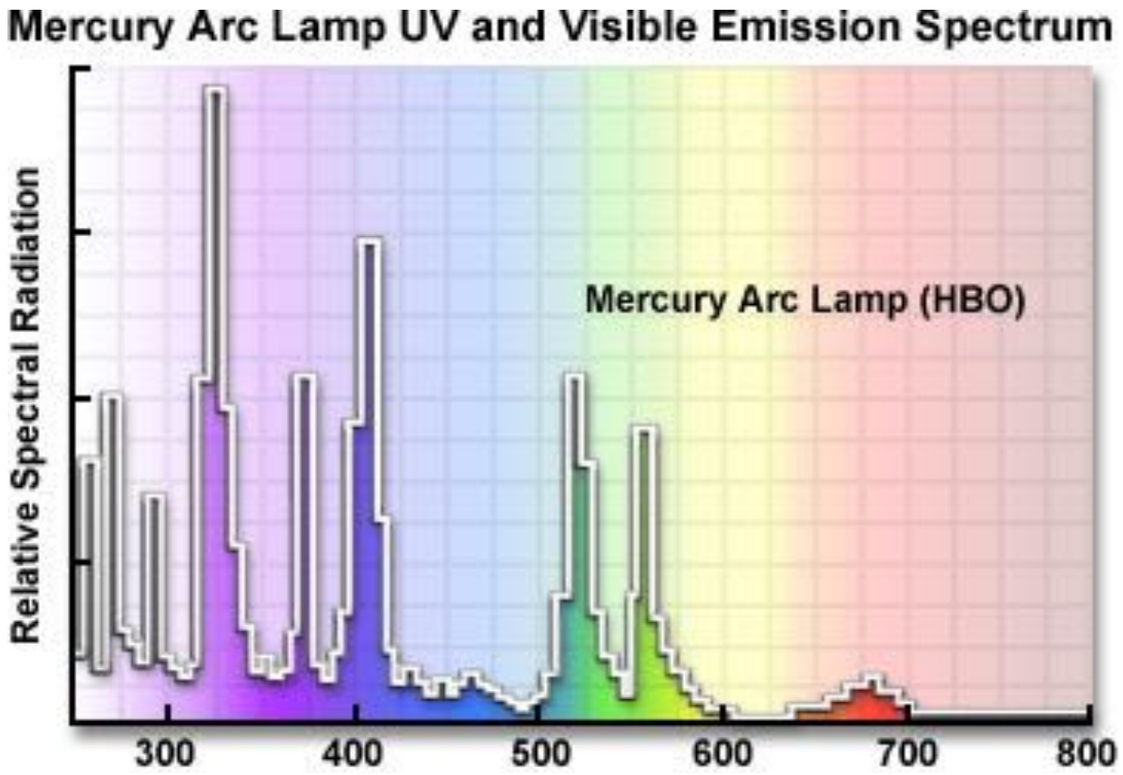


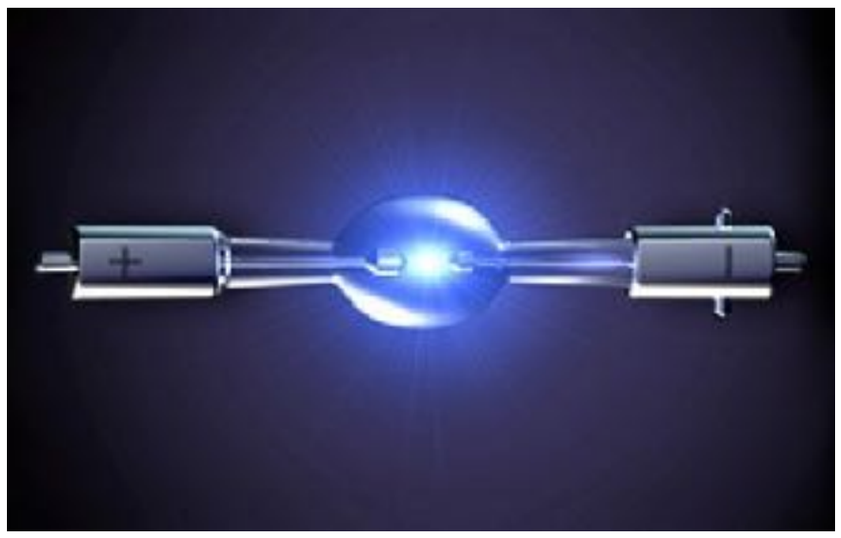
Illumination sources for widefield fluorescence microscopy

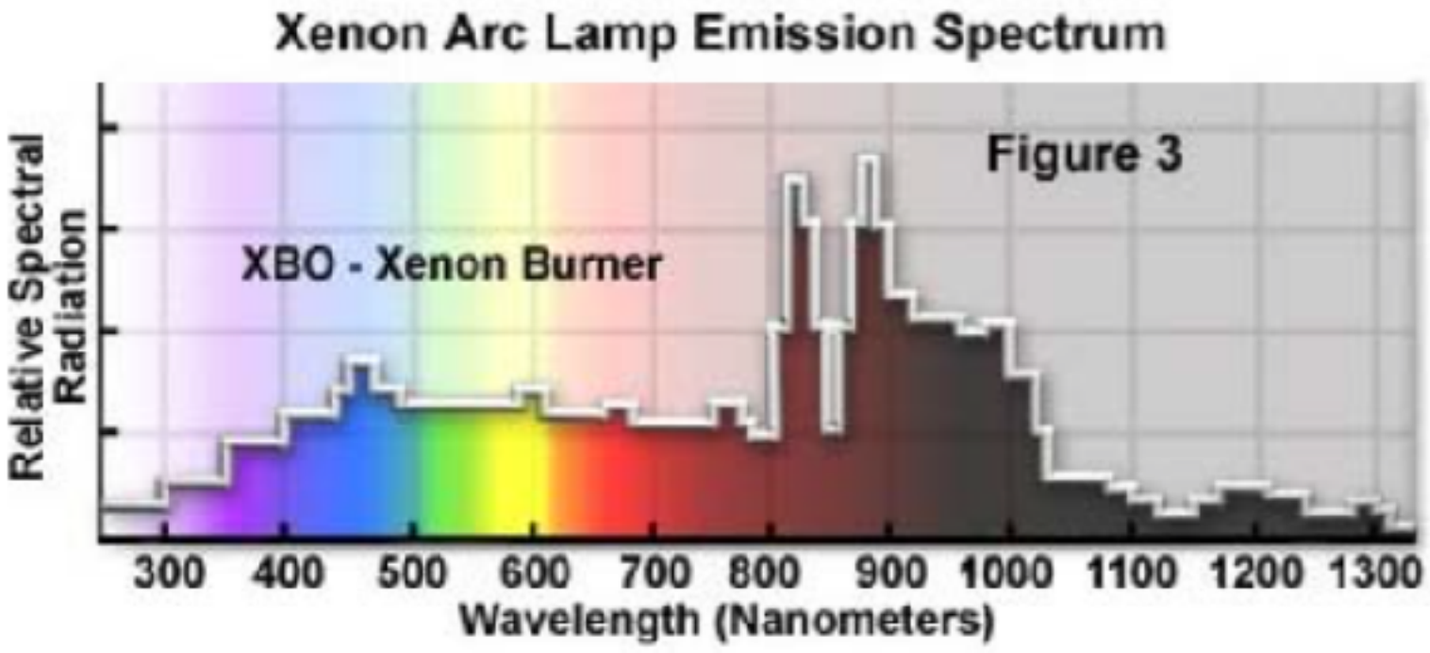
Widefield fluorescence

Arc Lamp Mercury

- 200h
- hazardous
- *out of use*





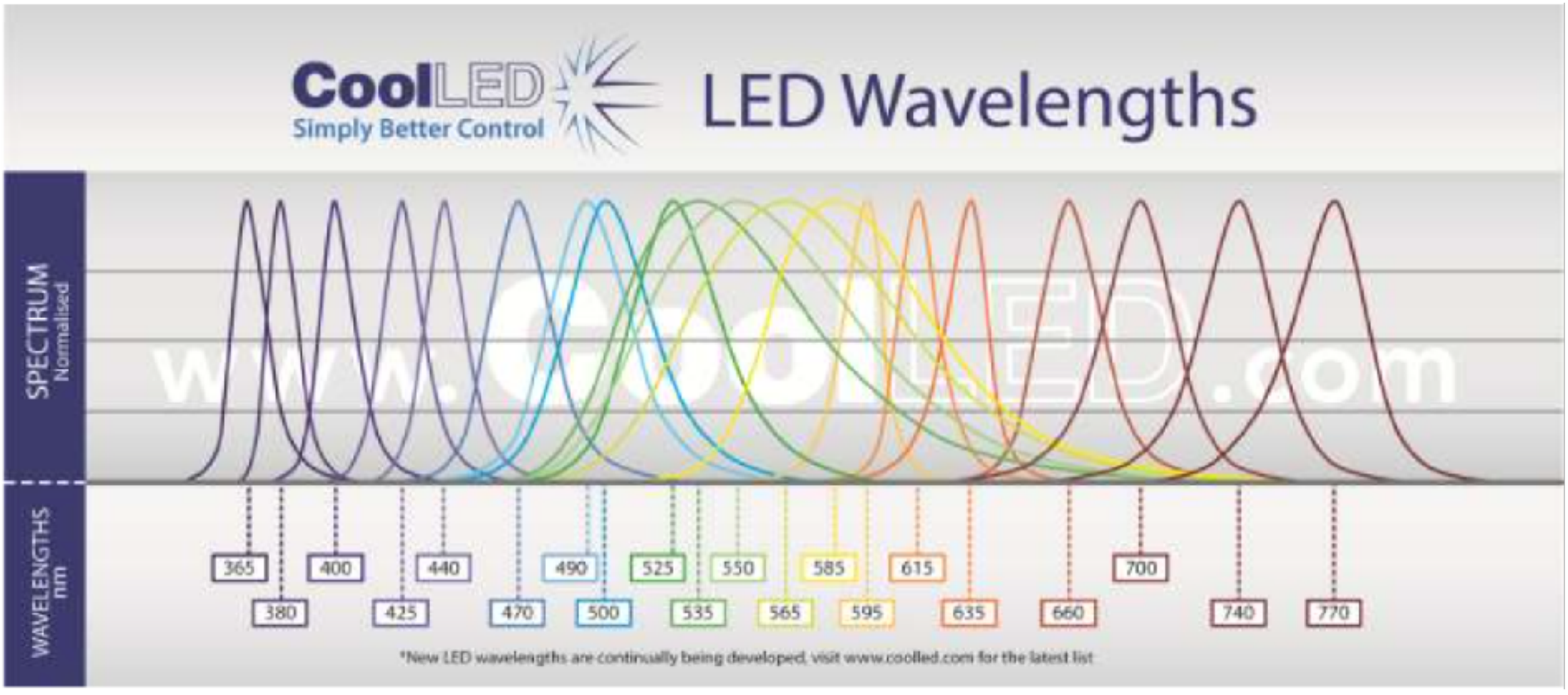


simultaneous excitation of multiple fluorophores over a wide wavelength range

Illumination sources for widefield fluorescence microscopy

State of the art for widefield fluorescence

LEDs Light Emitting Diodes



http://www.coolled.com/product-detail/led-wavelengths/

- Wide range of lines available
- 25,000 h

Illumination sources for fluorescence microscopy

Widefield **Confocal**

2-photon **TIRF**

Super-resolution

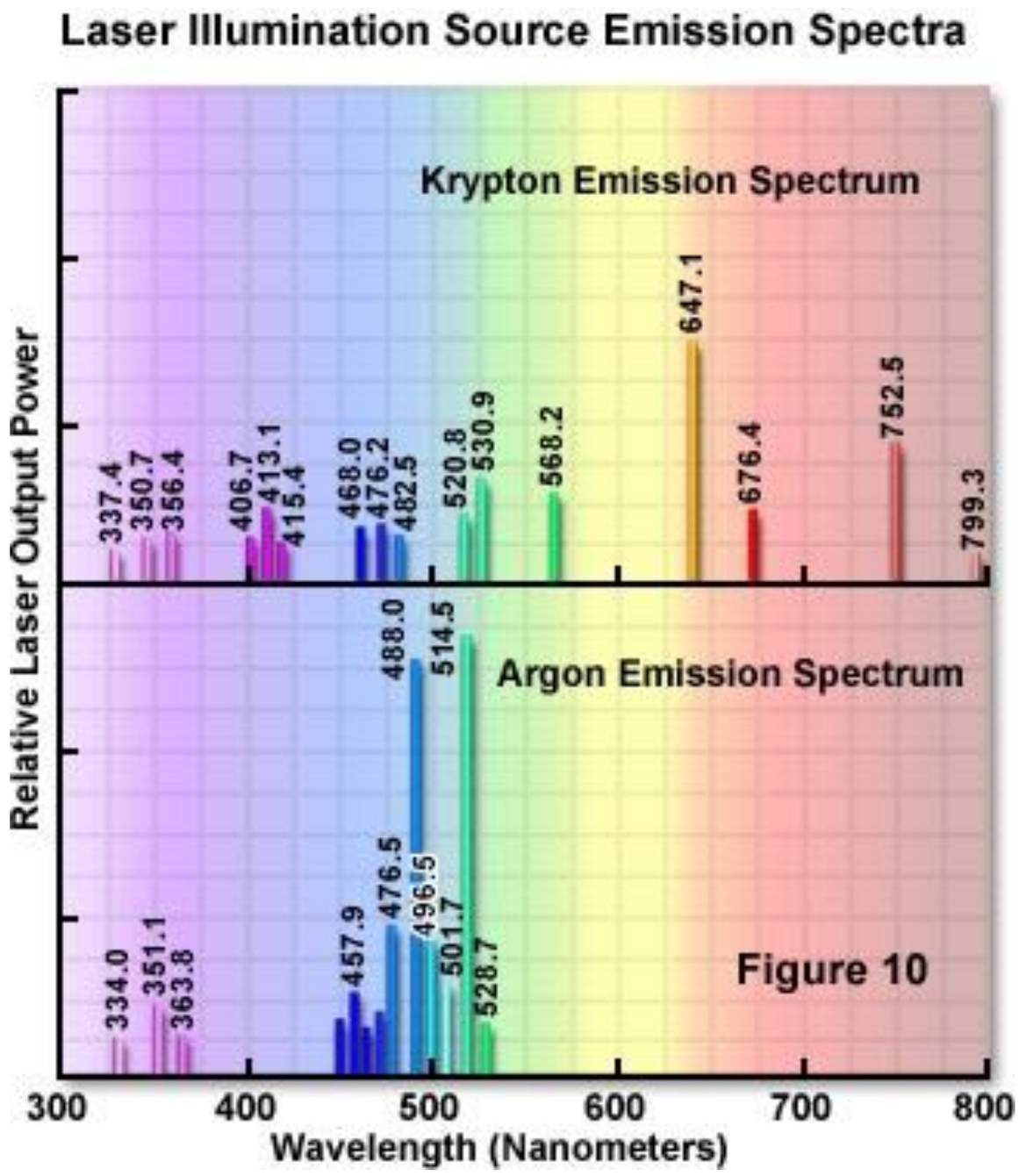
Only discrete lines!

lines	Alexa dye	
405	405	440
440	430	540
488	488	515
514	514	540
561	568	605
633	633	645

Narrow beams of highly monochromatic, coherent and collimated light

*** Diode lasers** *** Solid State lasers**

*** Gas lasers**



Basics of sample preparation

Fixed samples

in vivo (Lecture 7)

Why work with fixed material?

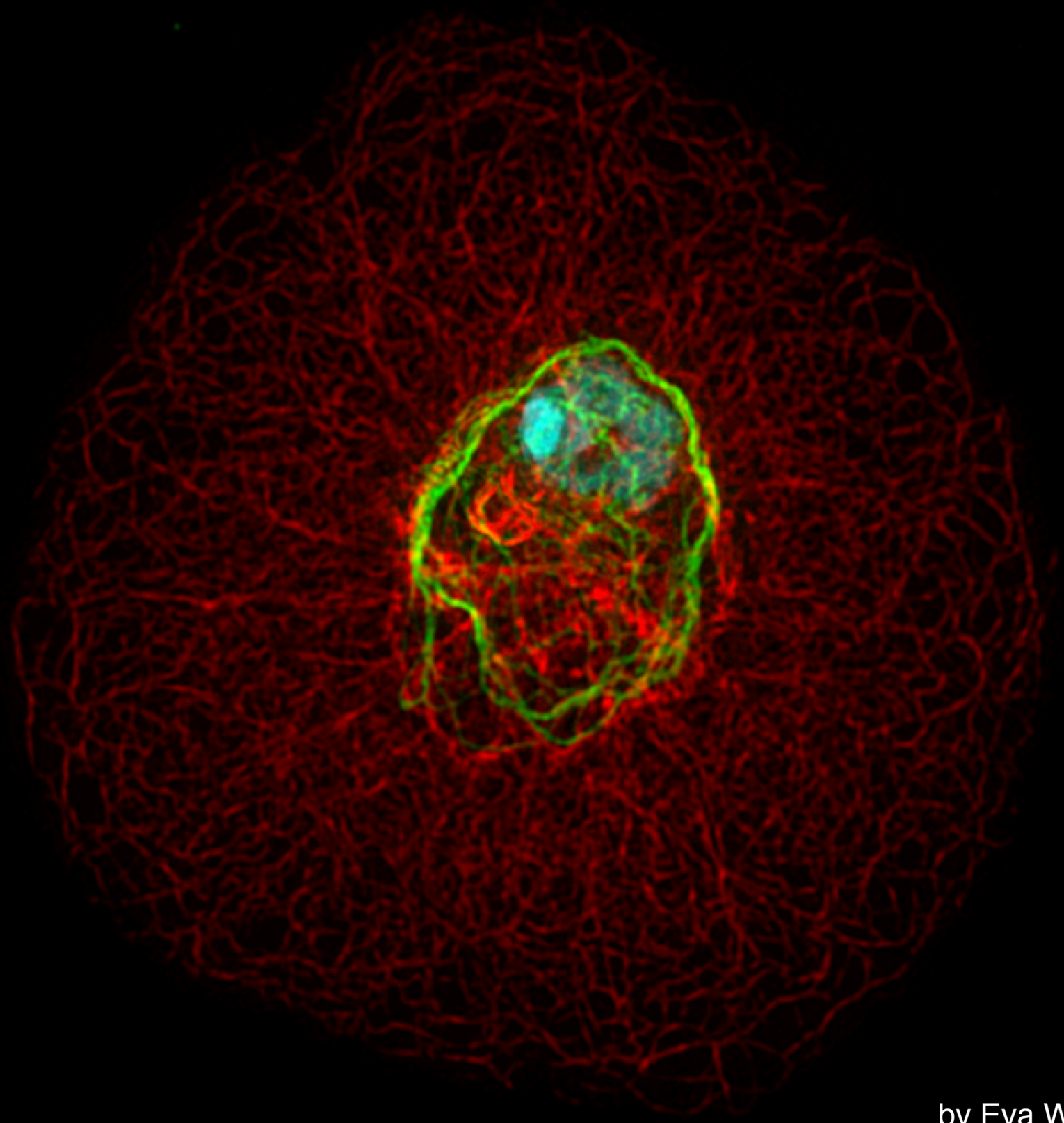
1. Convenience / Throughput

2. Widely applicable molecular labeling:
   Immunofluorescence
   FISH

3. Ease of multiplexing bright stable labels

Typical Immunocytochemistry Protocol

Fixation
Permeabilisation
Washes
Blocking
1° antibody
Washes
2° antibody
Washes
Mounting



by Eva Wegel

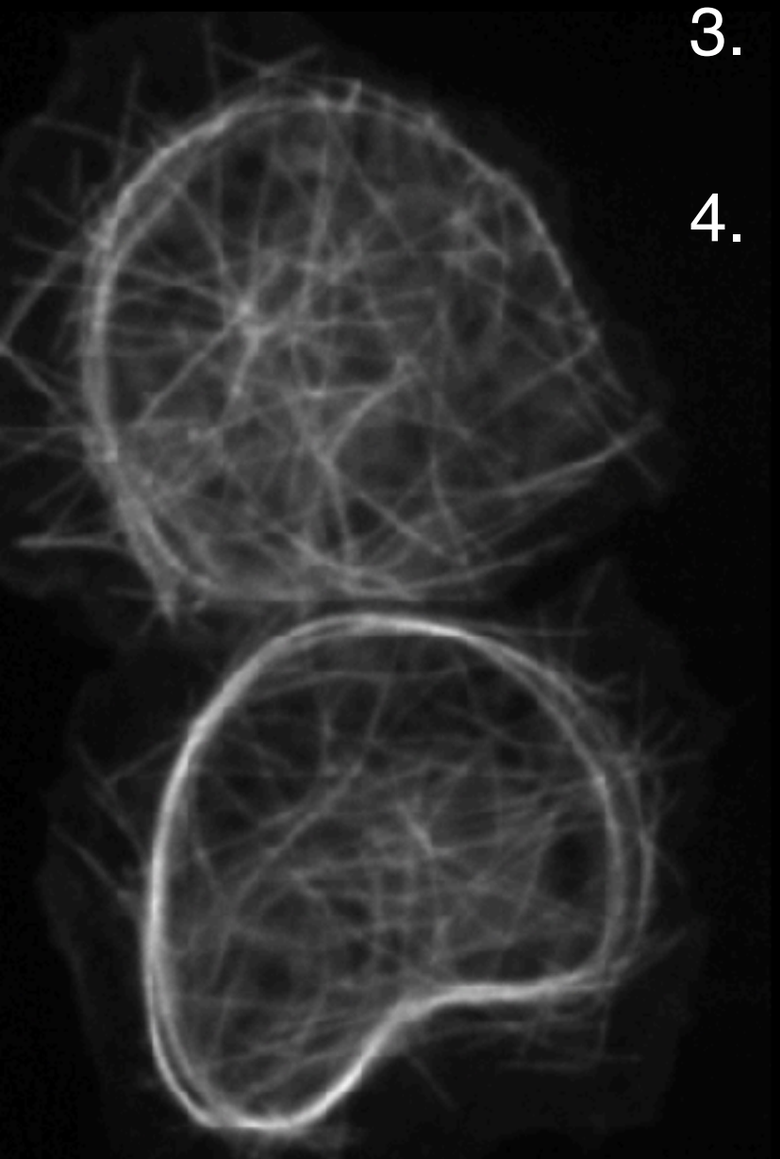
Immobilising the specimen

Sample holder must be suitable for imaging

by Esther Garcia

Fixation: preservation of cells or tissue in a life-like state

1. Preserve structural features
2. Uniform fixation throughout the sample
3. Enable dye labeling
4. Reduce background fluorescence

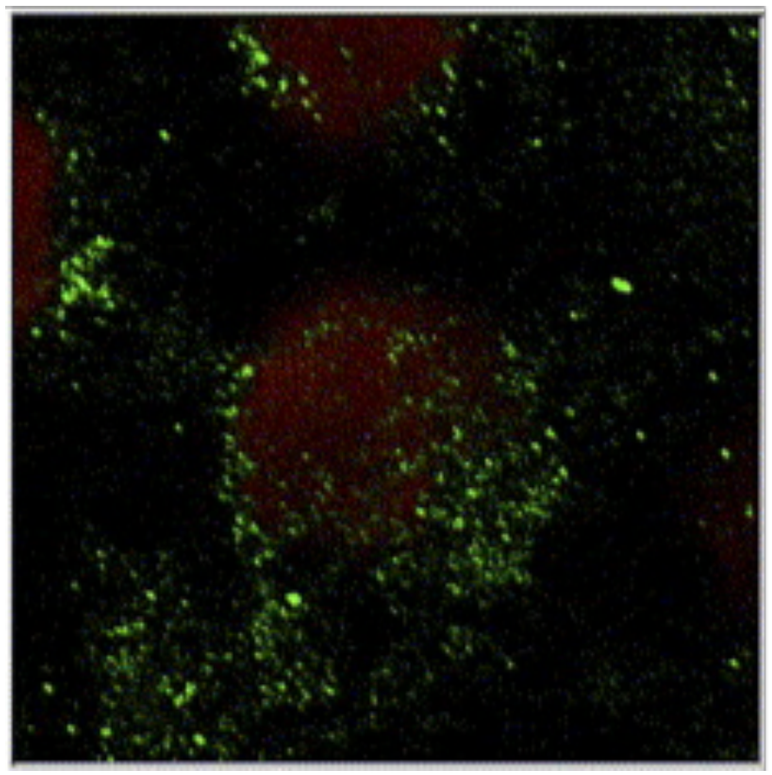


Microtubules in Drosophila macrophages
Left :
Live cells expressing Jupiter-GFP
Right:
PFA fixed cell stained with anti-tubulin antibody and Alexa Fluor 488

Types of Fixation

Denaturing fixation:

Cold methanol

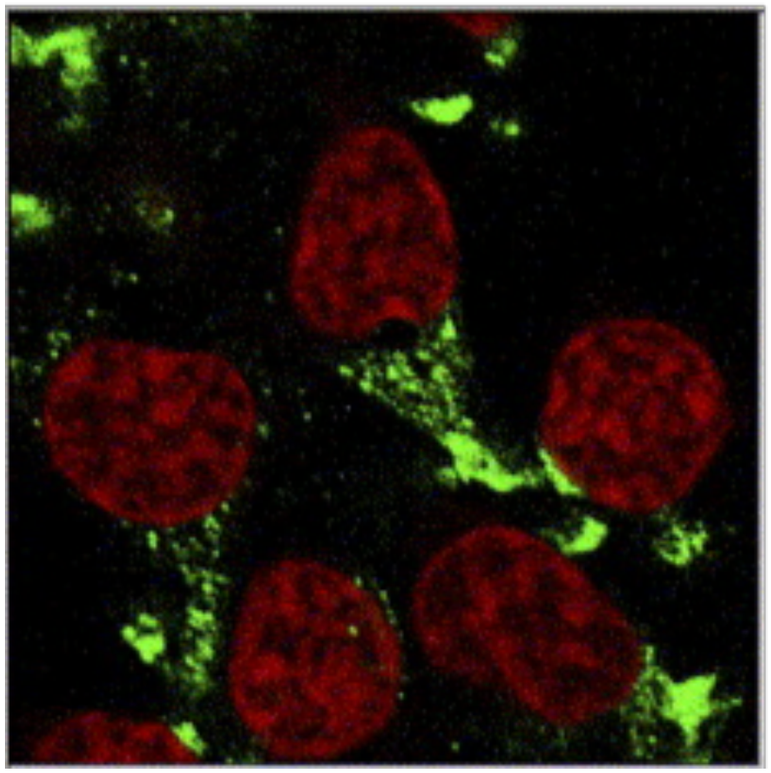


destroys 3D protein structure

dissolves lipids into micelles

Cross-linking fixation:

Formaldehyde (PFA)



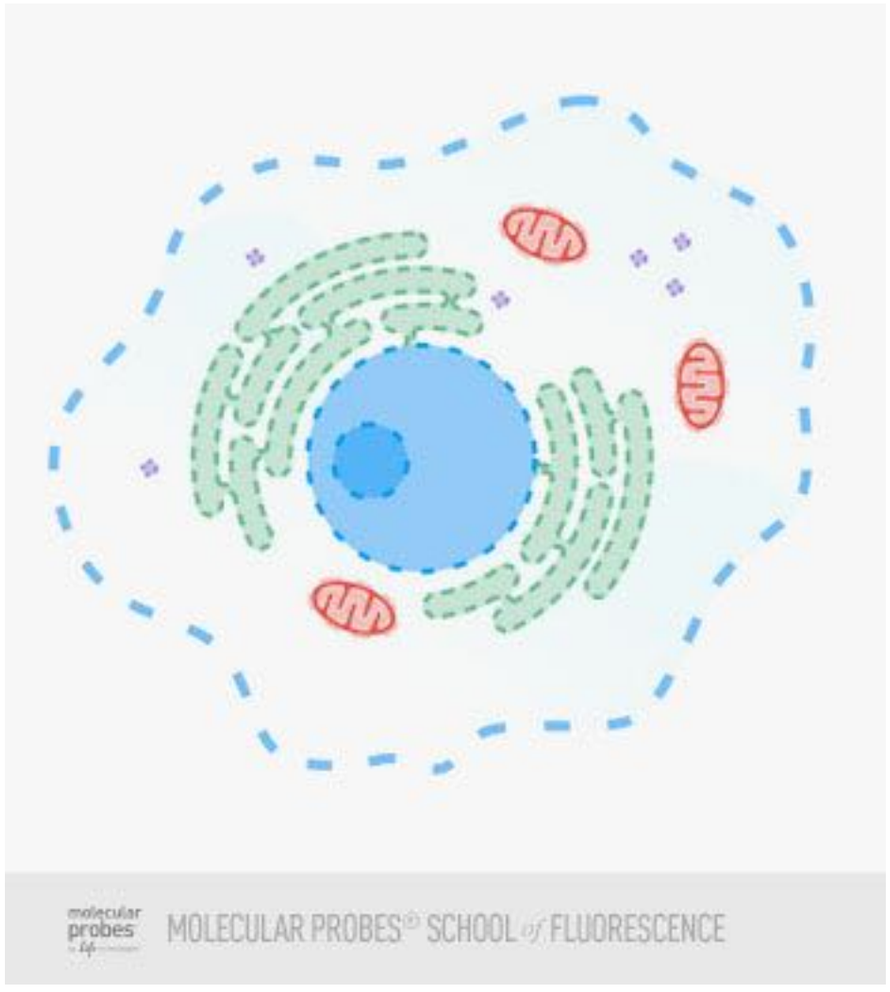
binds to proteins and some lipids,

but not RNA, DNA or most sugars

Sometimes a combination of both is necessary …

Permeabilisation

done by removing some lipids with detergents



Tween 20

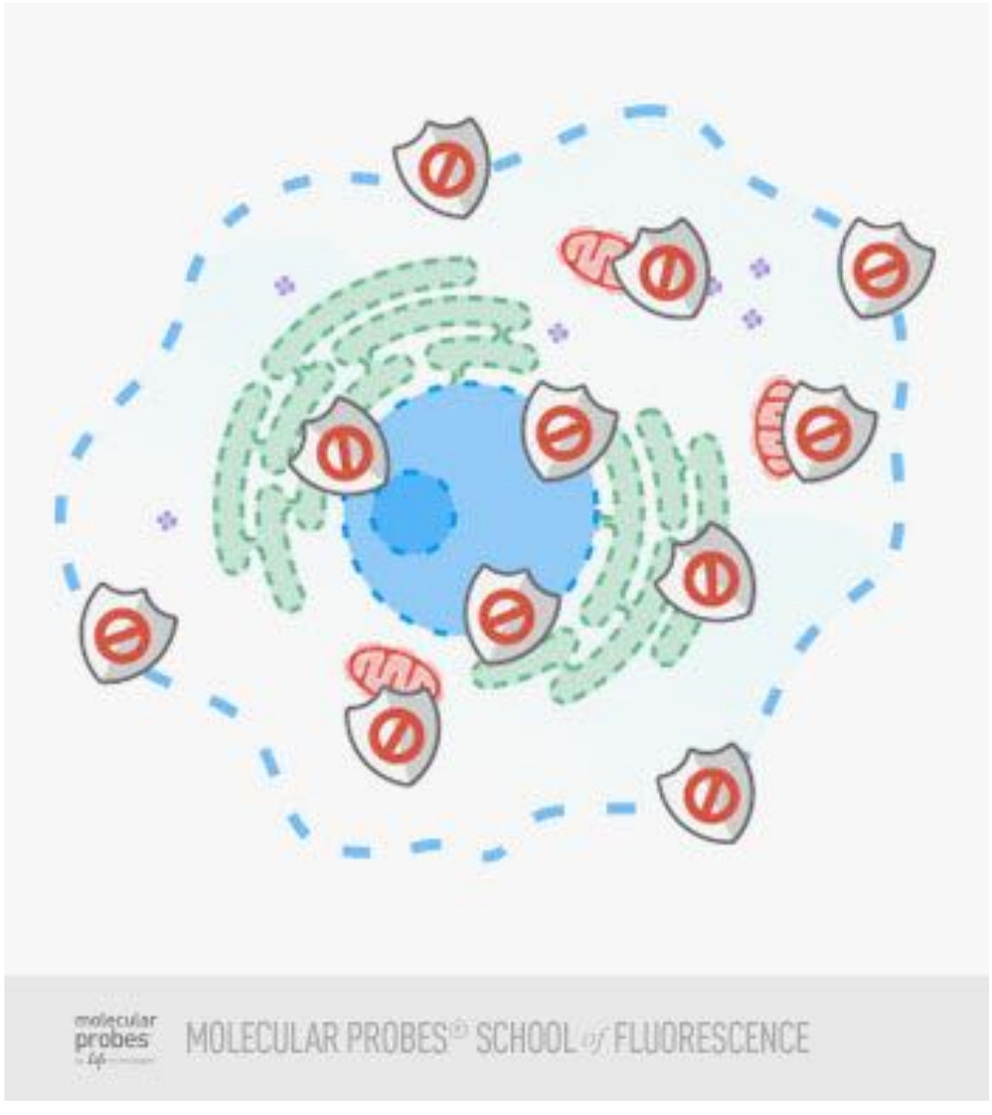
Triton X-100

To allow large labels (antibodies) to penetrate fixed cells/tissue

Blocking

Reduction of nonspecific “background” staining

done with a solution containing excess of protein



Bovine Serum Albumin (BSA)

Casein (or non-fat dry milk)

blocking proteins prevent low-affinity antibody interactions elsewhere in the sample

Immunolabeling (antibodies)

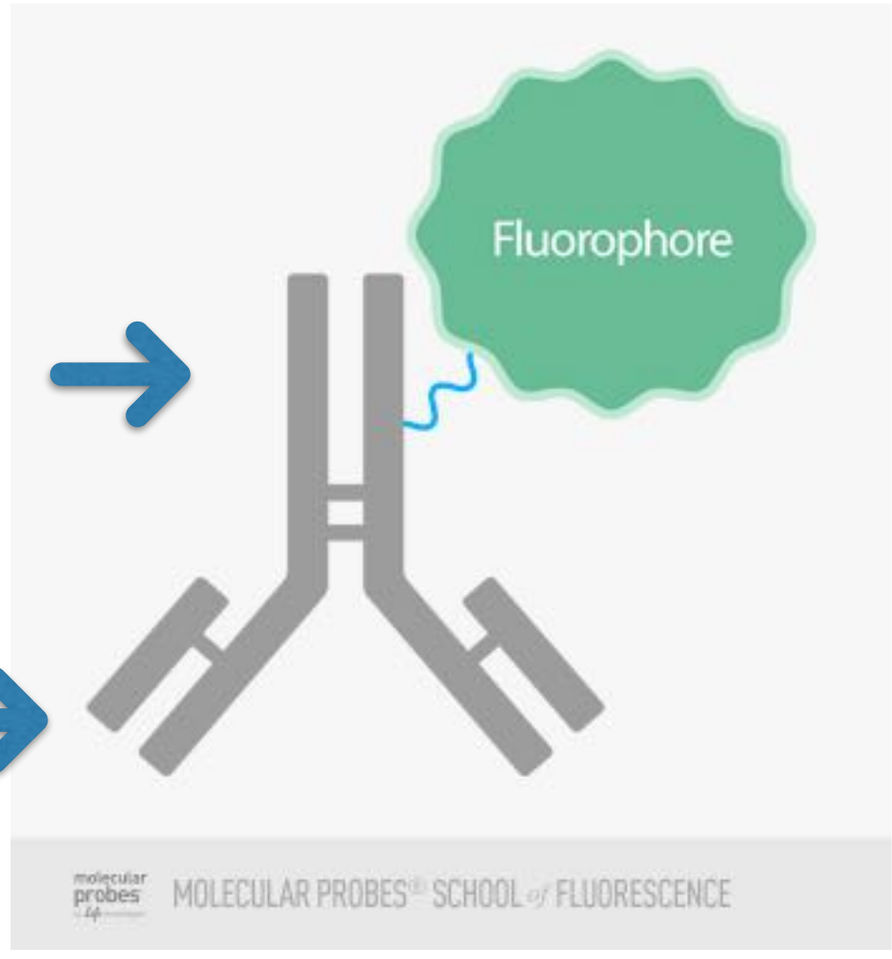
Antibody (large Y-shaped protein called immunoglobulin)

produced by the immune system, found in the blood or other body fluids of **vertebrates**.

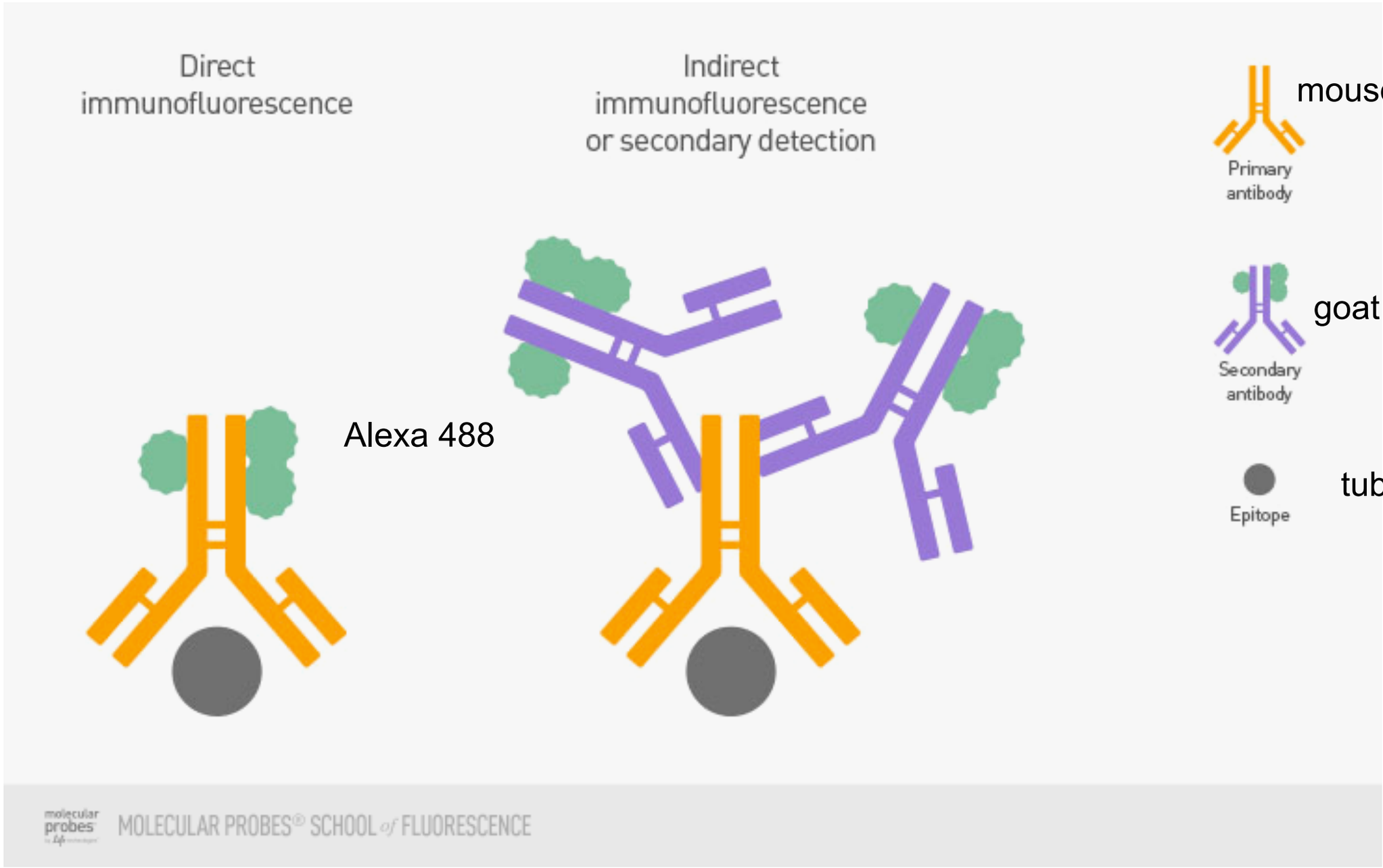
The **antibody** recognises unique parts of the foreign target called an **antigen**.

Conserved among species

VARIABLE
Antigen binding site



Immunolabeling (antibodies)



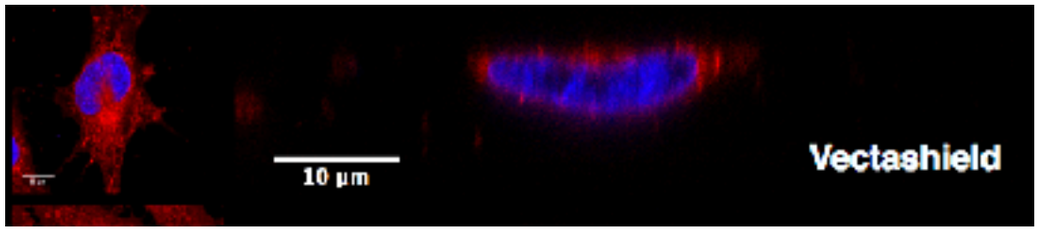
mouse anti-tubulin

goat anti-mouse

tubulin

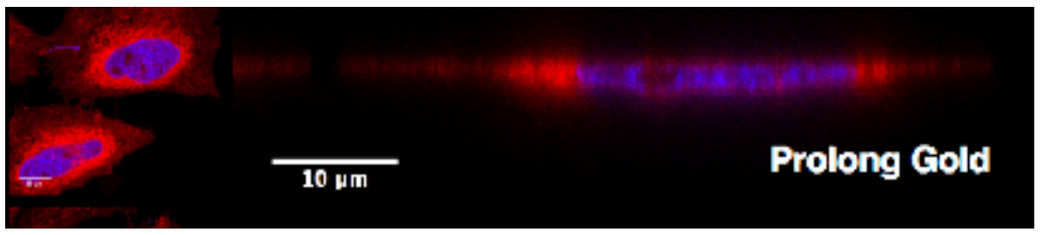
Mounting

- Non-hardening



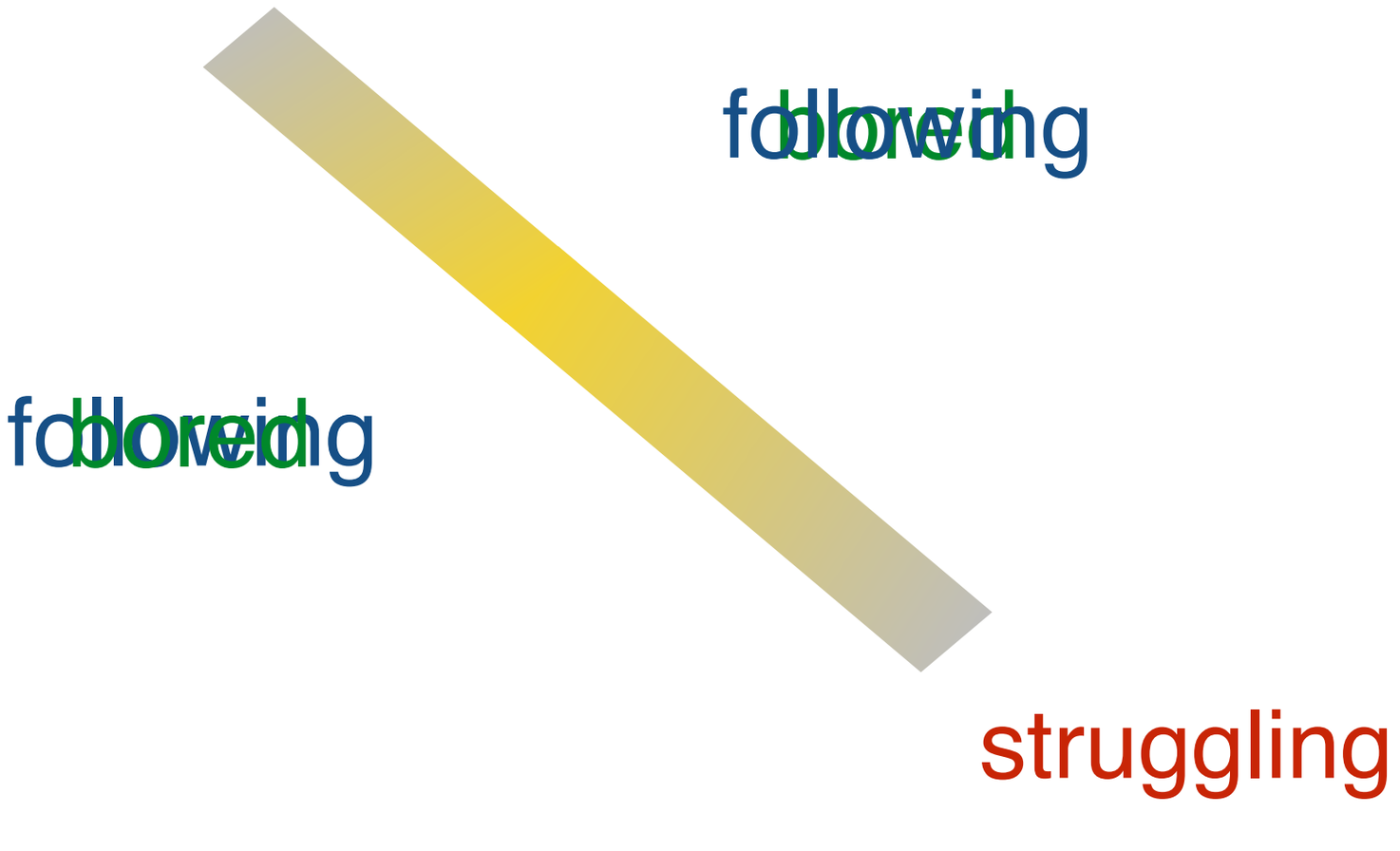
  - Short-term storage (days to few weeks)
  - **Dabco, Glicerol, Vectashield** (antifading agent, but does not work with FarRed dyes)

- Hardening



  - Long term storage (months)
  - It can flatten the cell if polymerises too fast
  - **Prolong Gold, Vectashield harset**

Inflection point in the lecture



PSF (Point Spread Function) in fluorescence

Point Spread Function

PSF is a measure of the microscope response to a point source of light

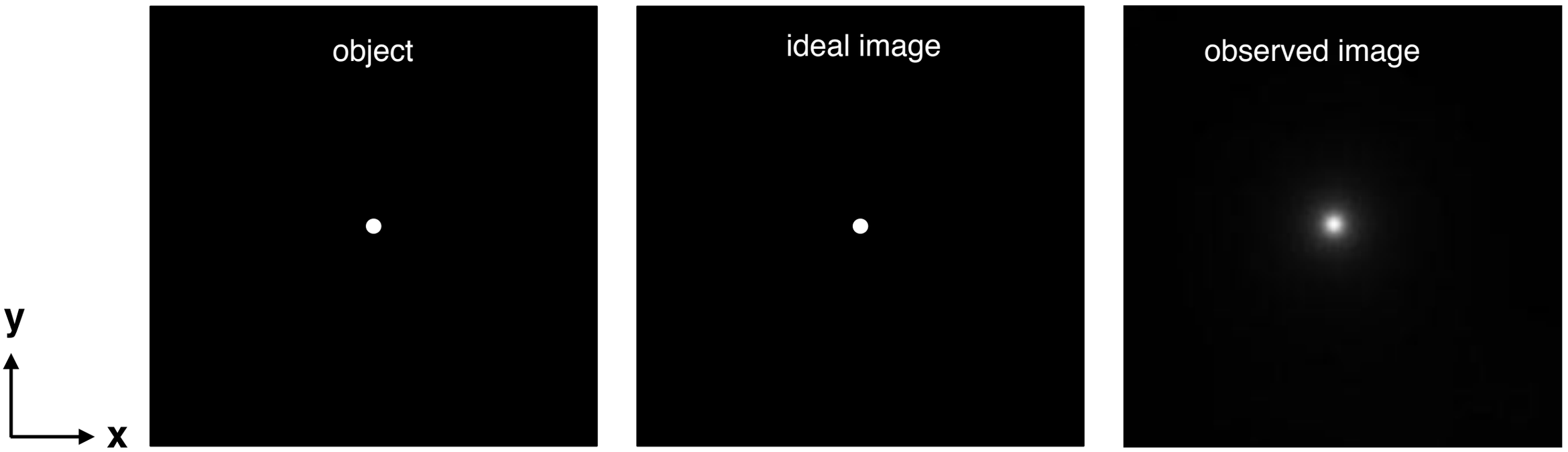
- microscope performance
- x, y, z info
- image quality
- alignment
- optical resolution

Why bother?

PSF (Point Spread Function) in fluorescence

Point Spread Function

How does light spread out from a single point?



Light is emitted in all directions

If all light was collected and if light would travel in straight lines

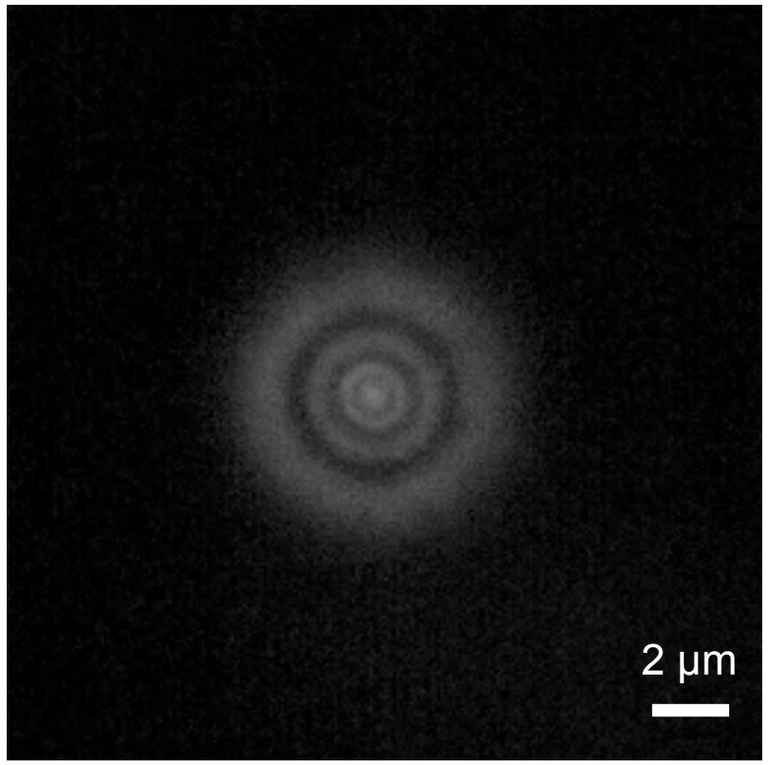
But the point actually looks blurred / distorted because of diffraction (Airy diffraction pattern)

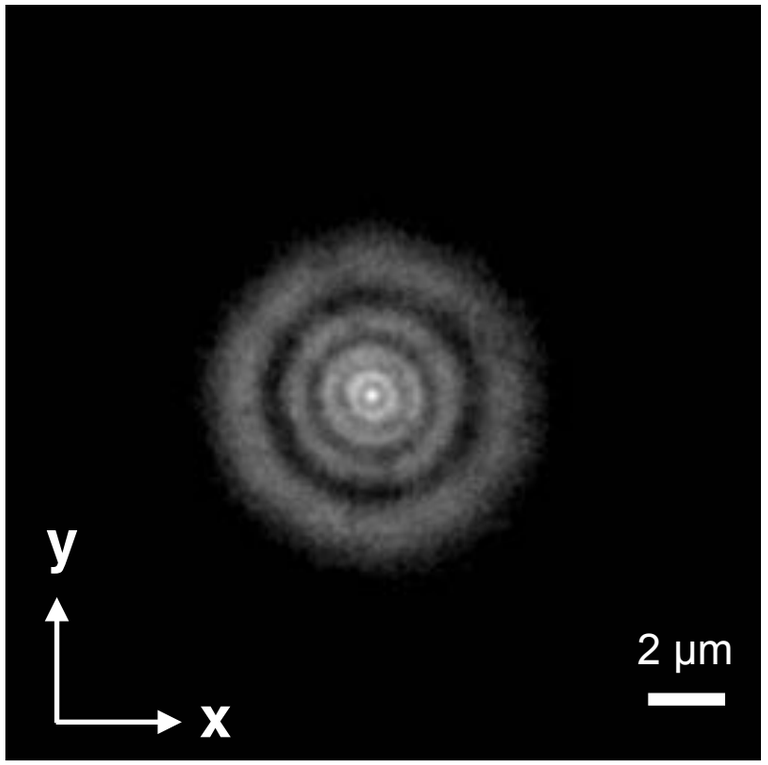
Fluorescent bead, single dye, or a fluorescent protein as a point source of light

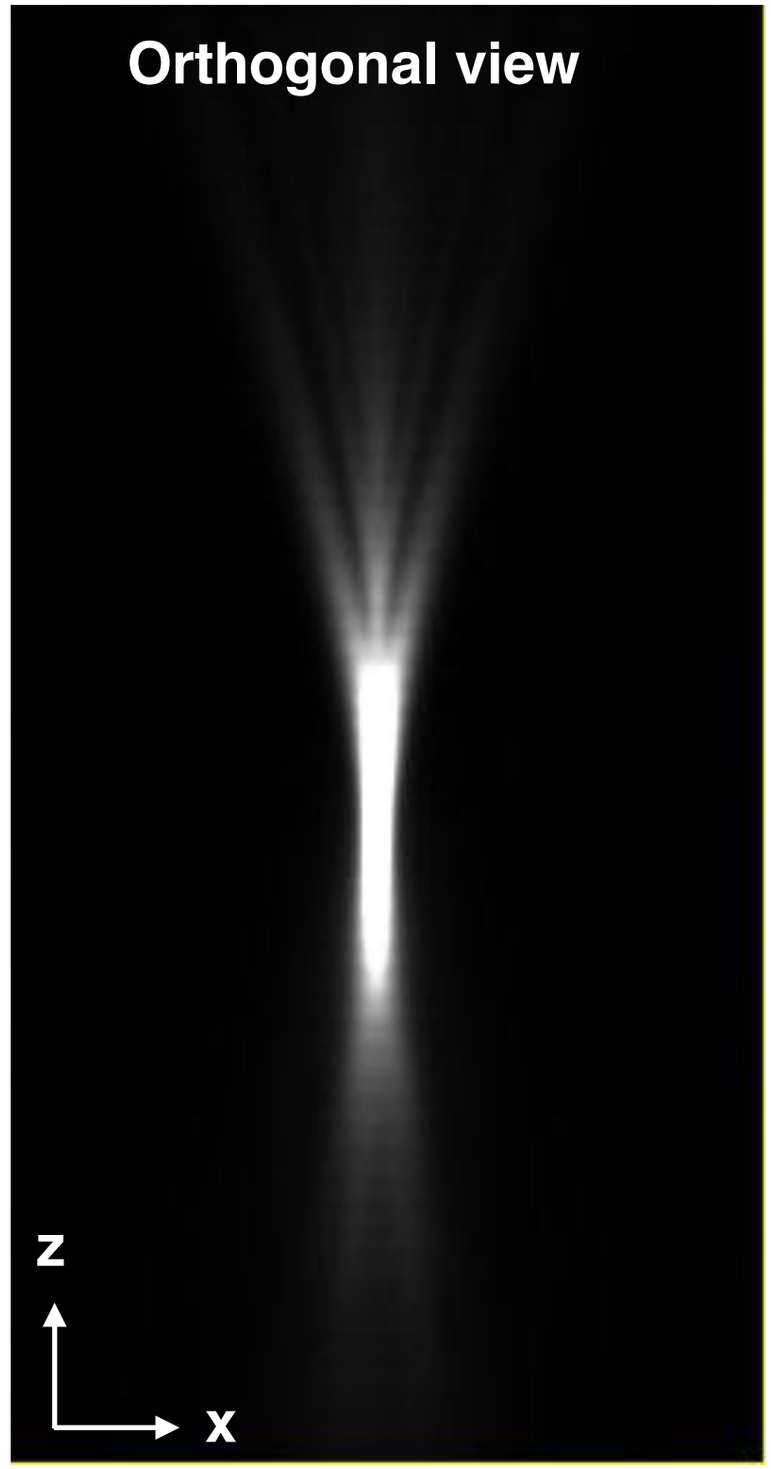
PSF (Point Spread Function) in fluorescence

PSF

red fluorescent 170 nm bead







Airy disk diffraction pattern

(concentric rings)

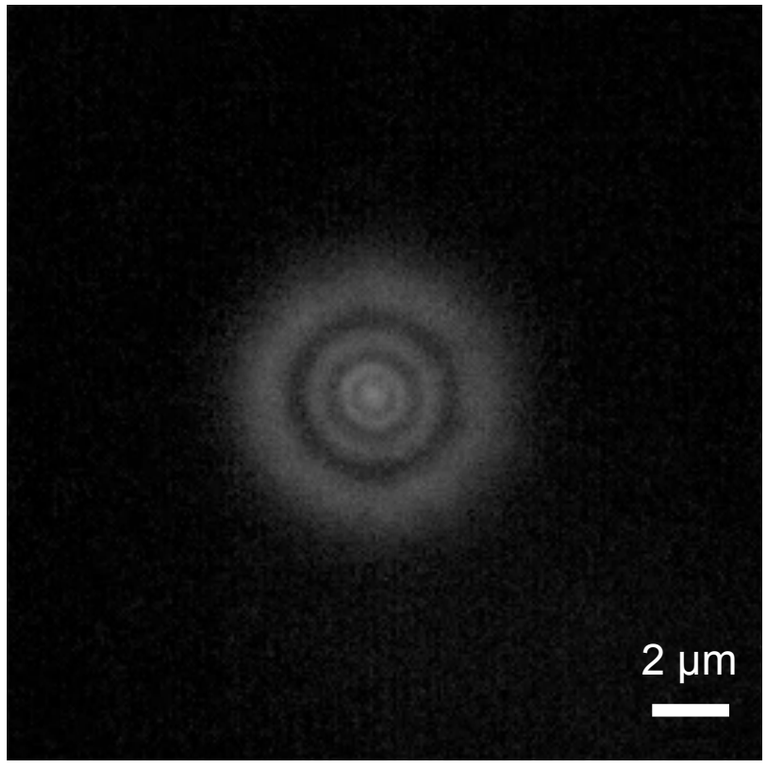
Light waves emitted from a point source are not focused into an infinitely small point by the objective

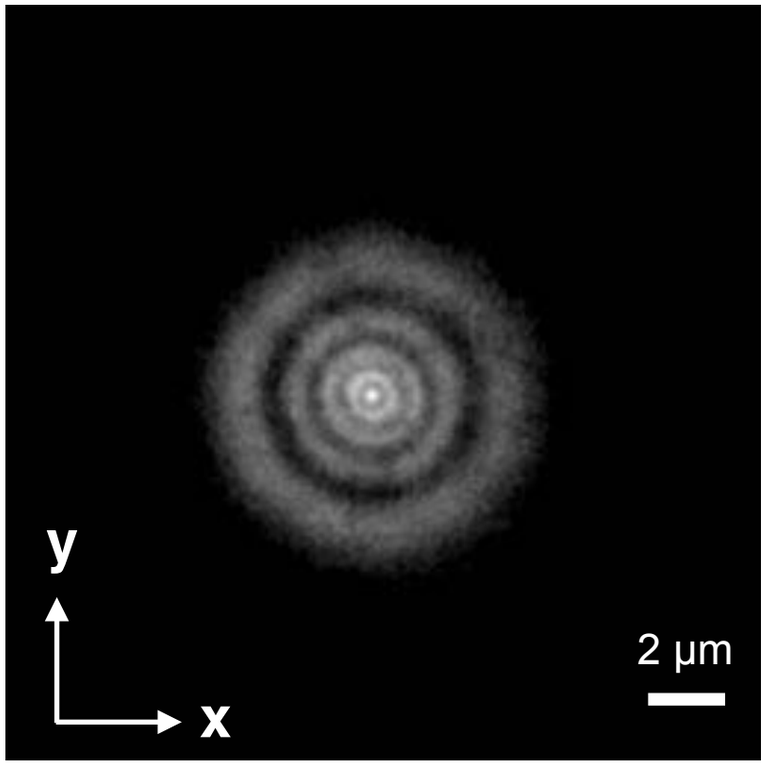
They converge together and interfere in the image plane

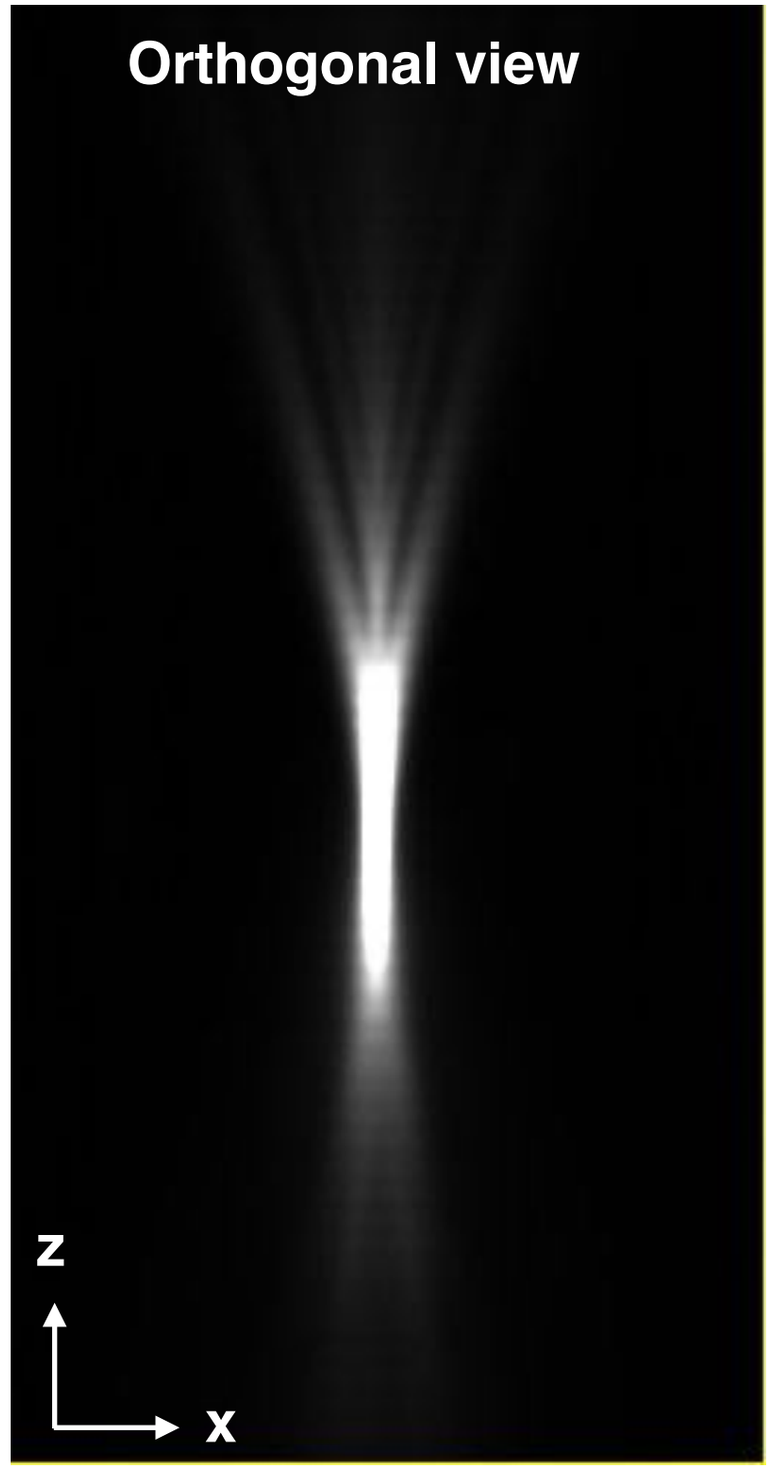
PSF is the 3D image of a point-like object under the microscope

PSF

red fluorescent 100 nm bead







What can we observe?

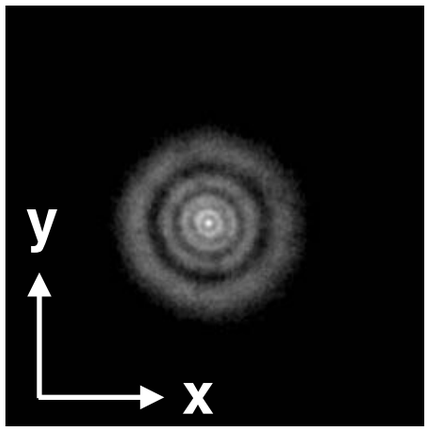
- Blur is broader in z than xy *RESOLUTION*
- How symmetric is the distribution

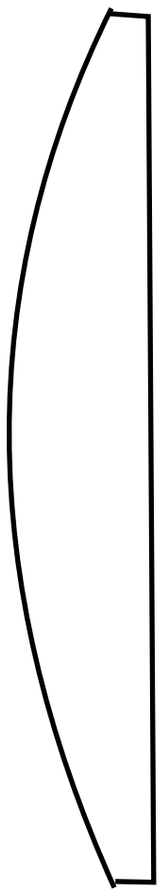
ALIGNMENT, SPHERICAL ABERRATIONS, MISMATCH REFRACTIVE INDEX

Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane

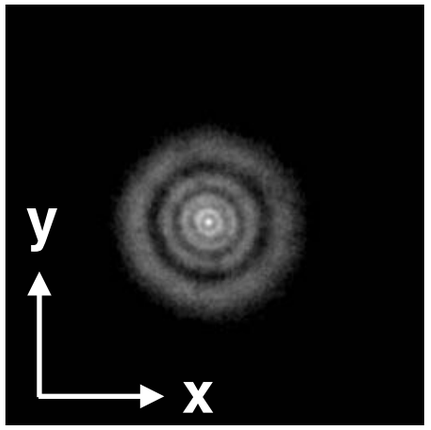


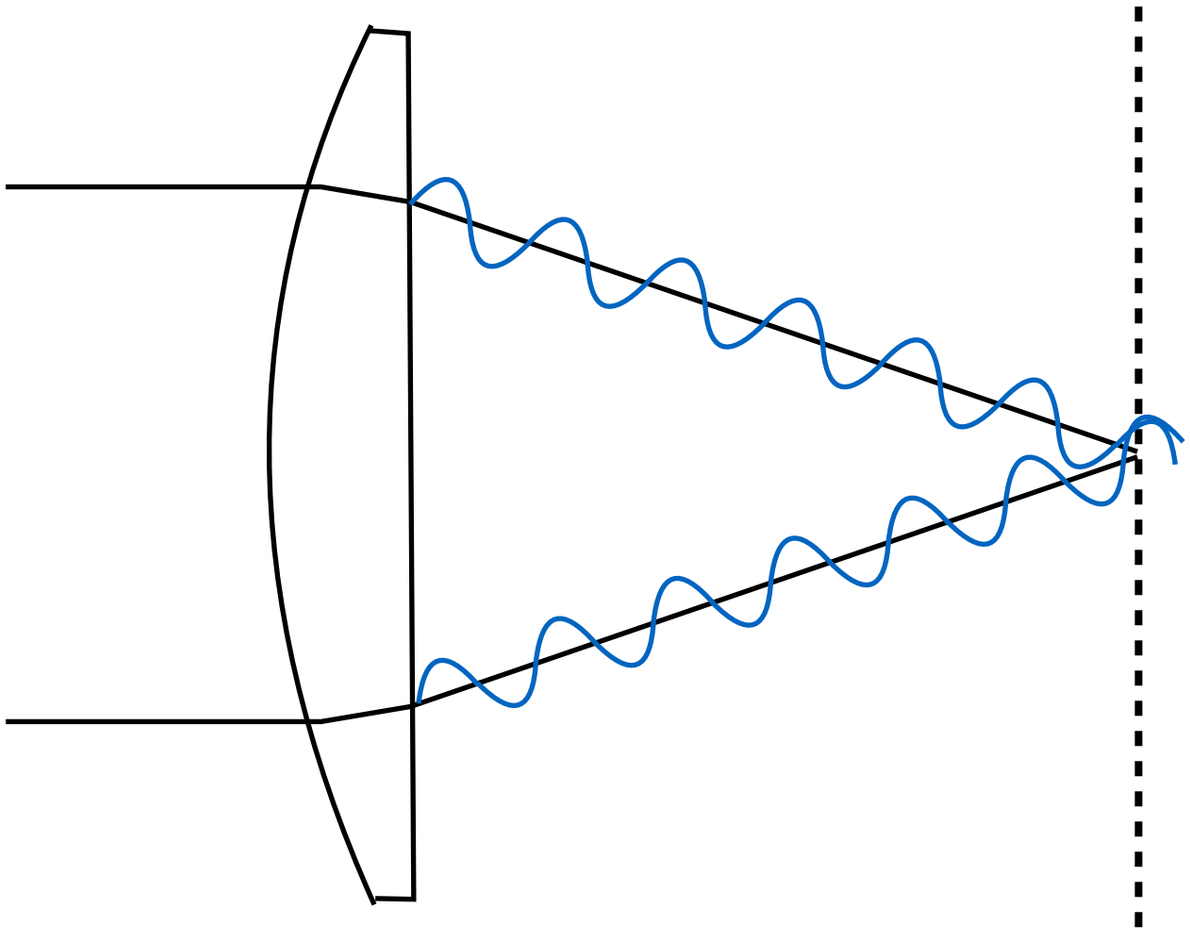


Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane



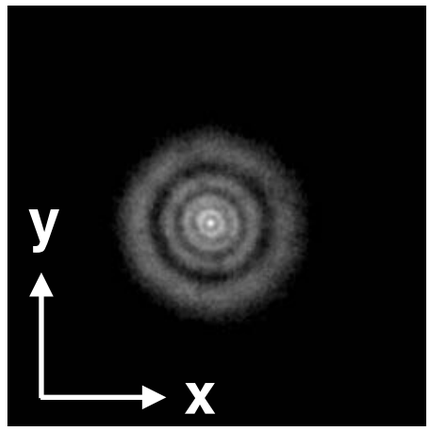


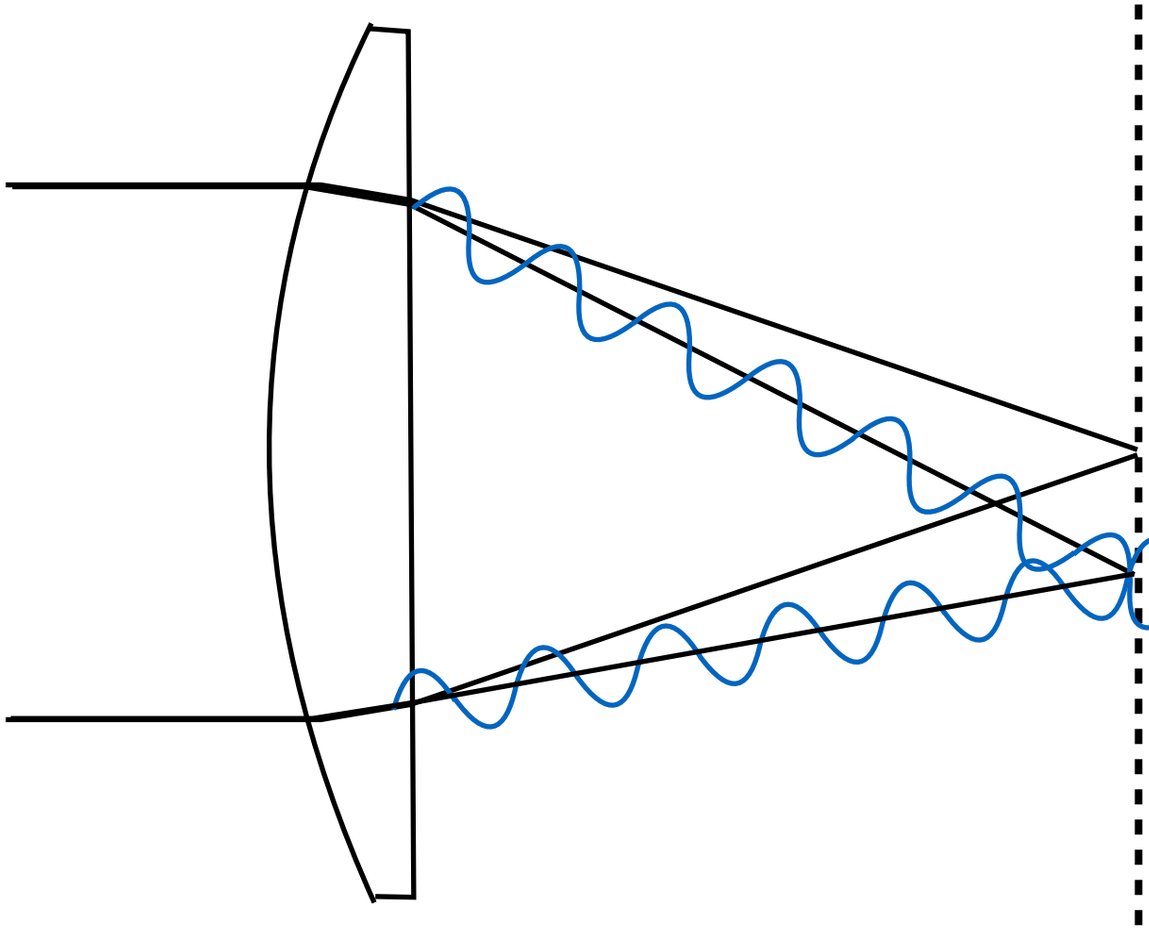
in phase - Constructive interference

Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane





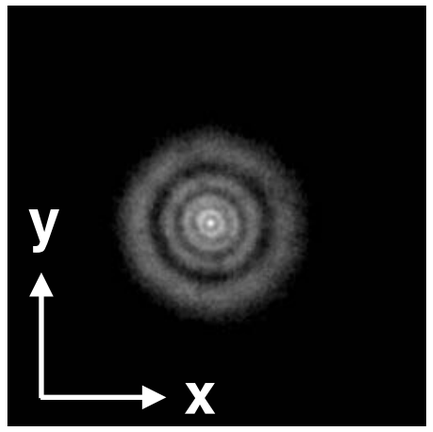
in phase - Constructive interference

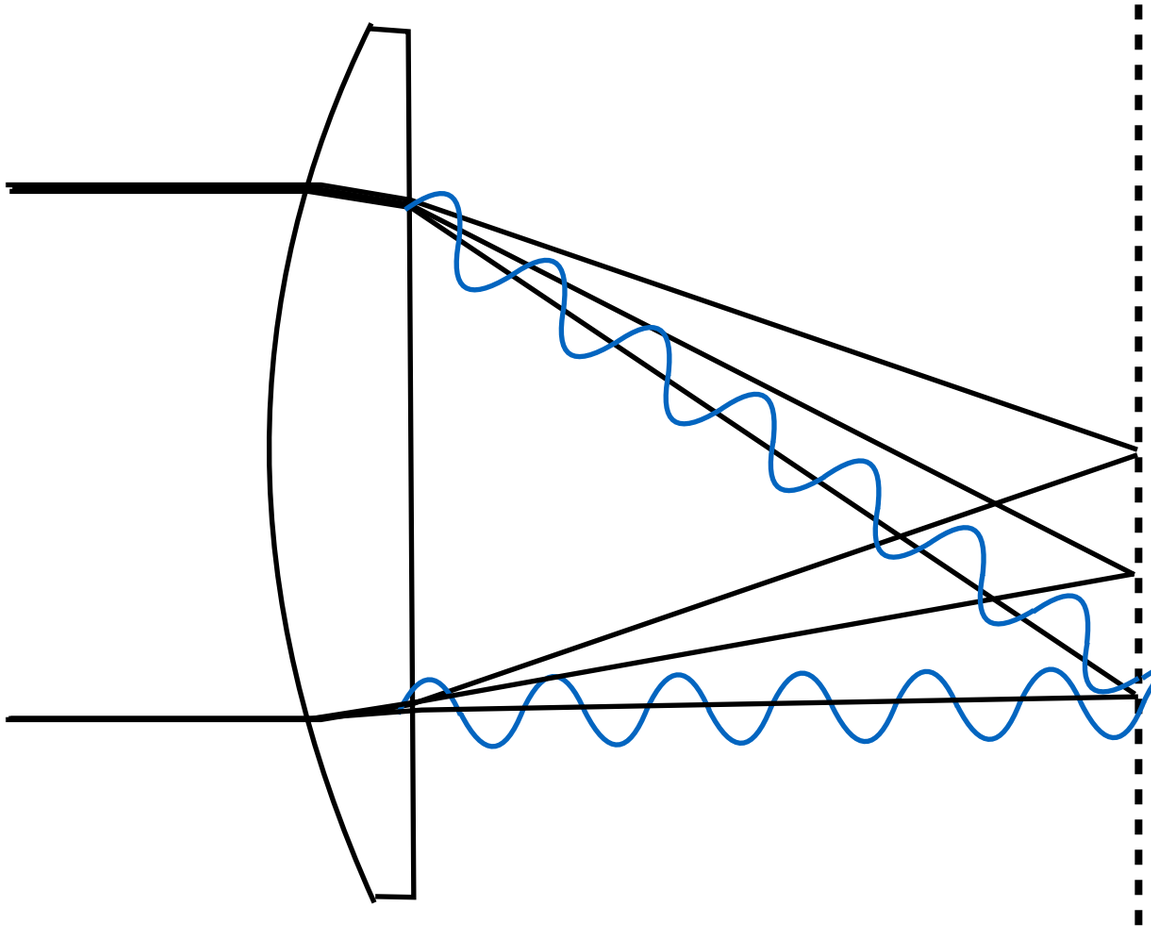
out of phase - Destructive interference

Why blurred and how is the Airy diffraction pattern generated?

Objective lens

Image plane





in phase - Constructive interference

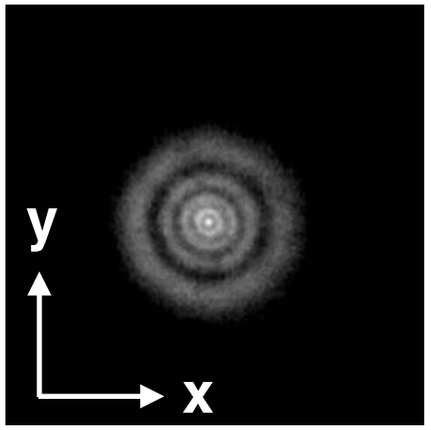
out of phase - Destructive interference

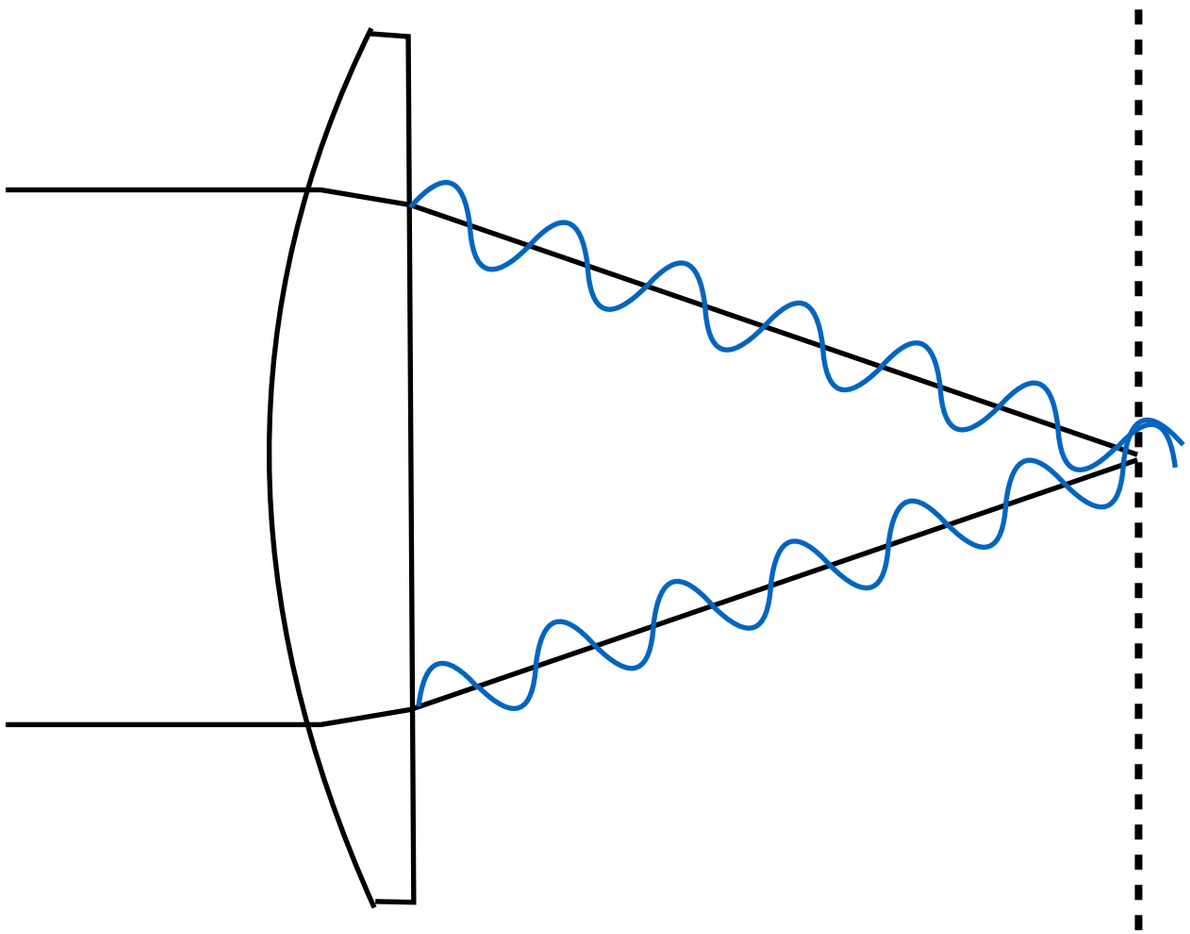
in phase - Constructive interference

What does depend on…?

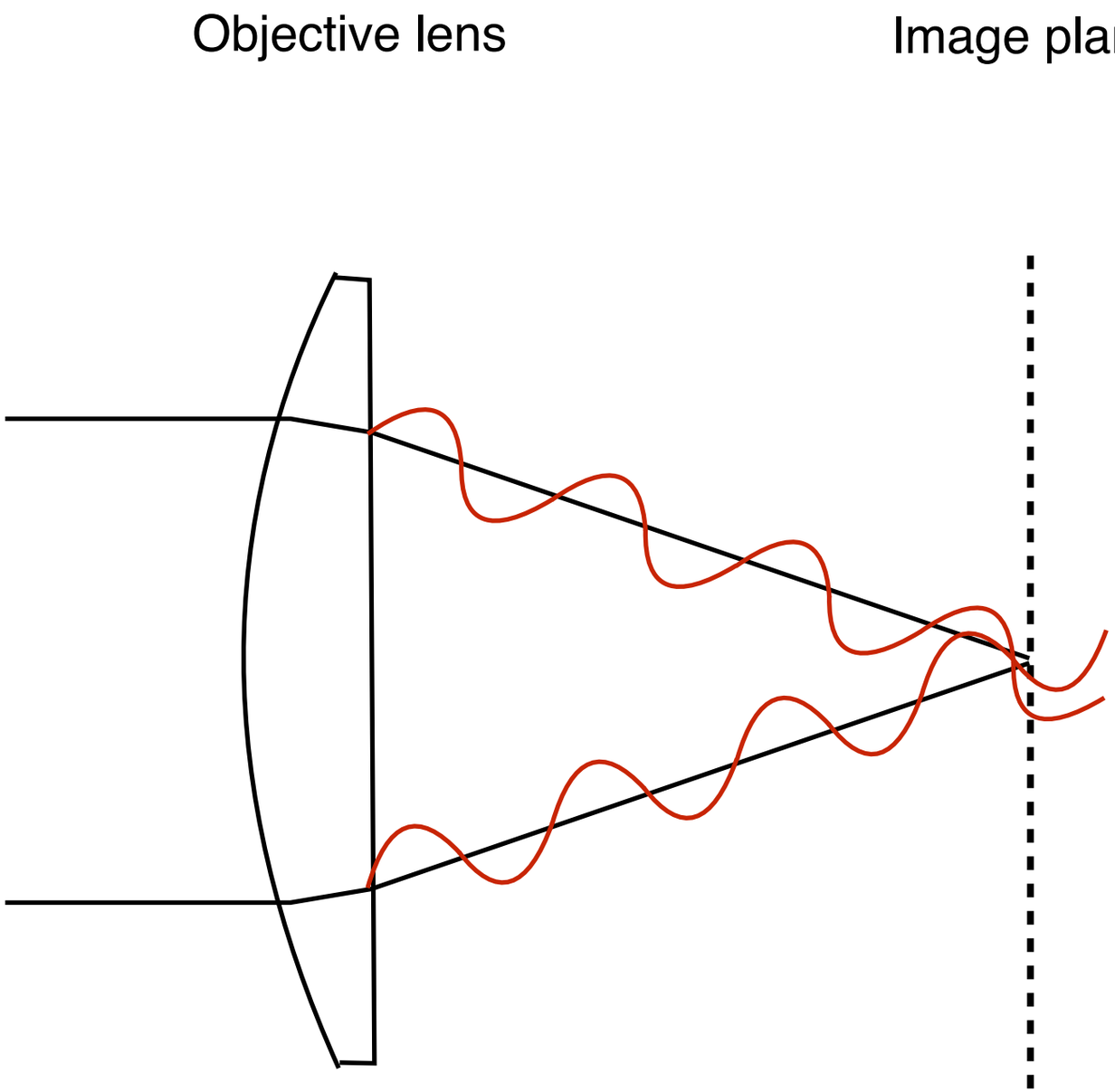
Objective lens

Image plane





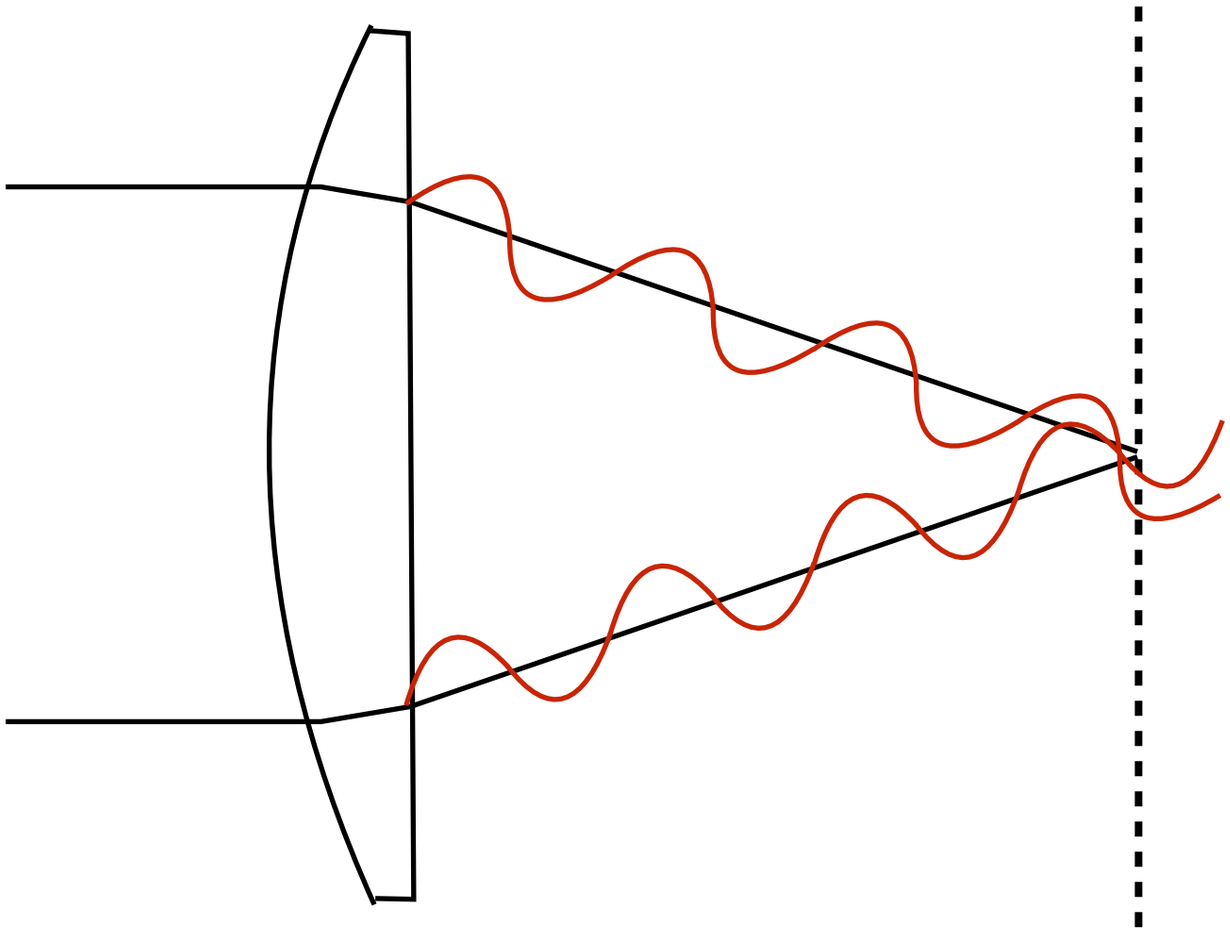
What does depend on…?



What does depend on…?

Objective lens

Image plane



Numerical aperture

What does depend on…?

Objective lens

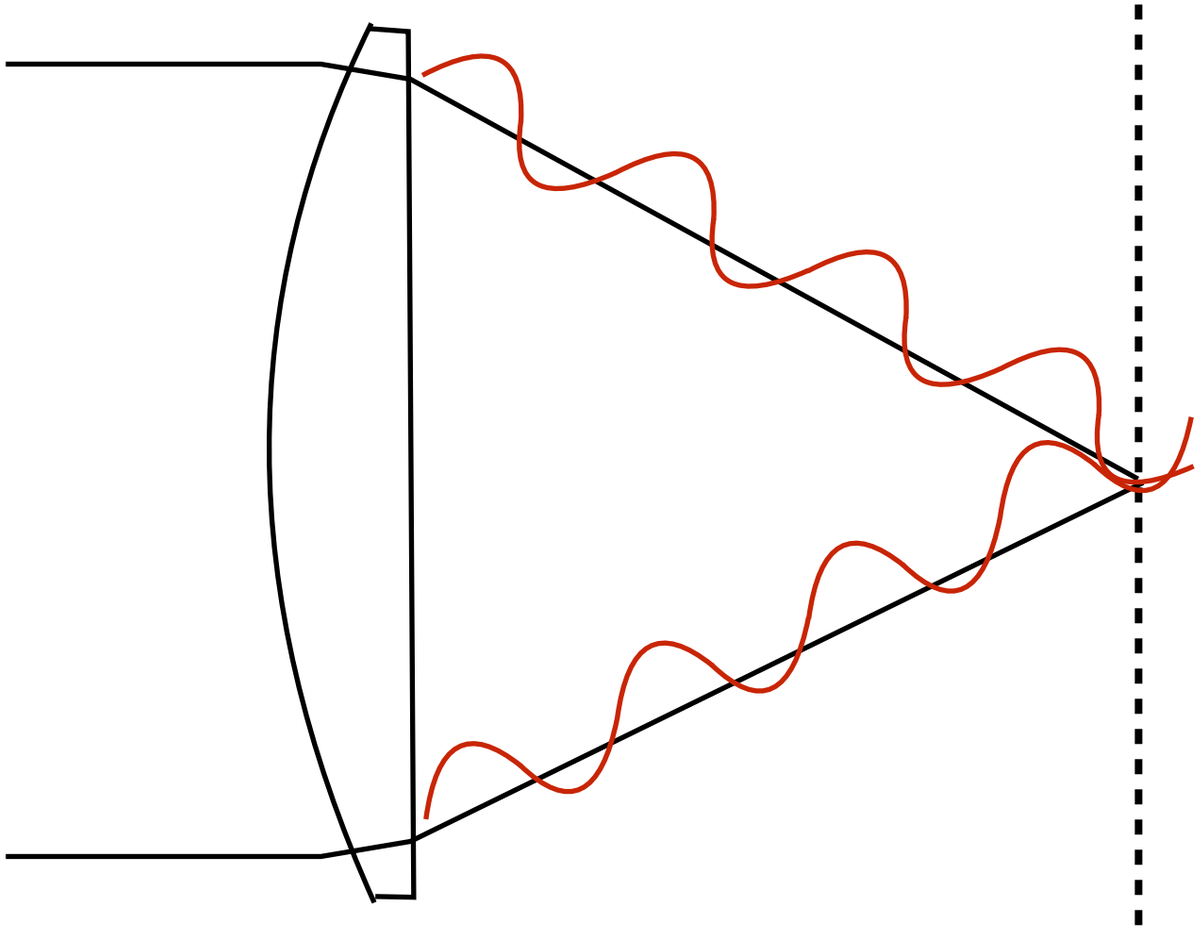
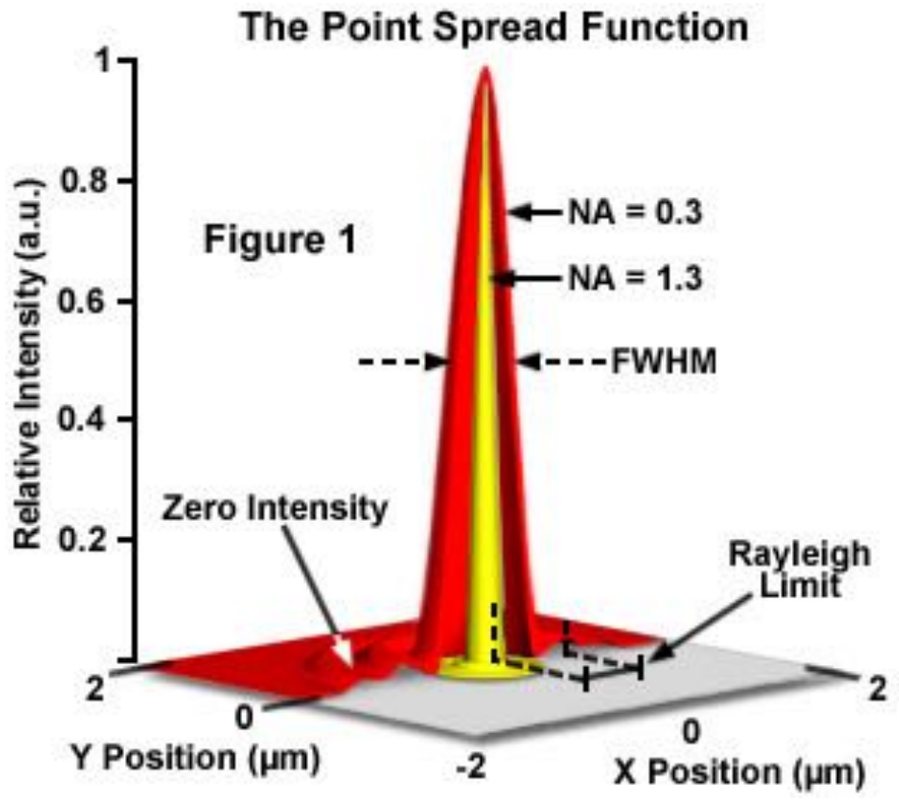
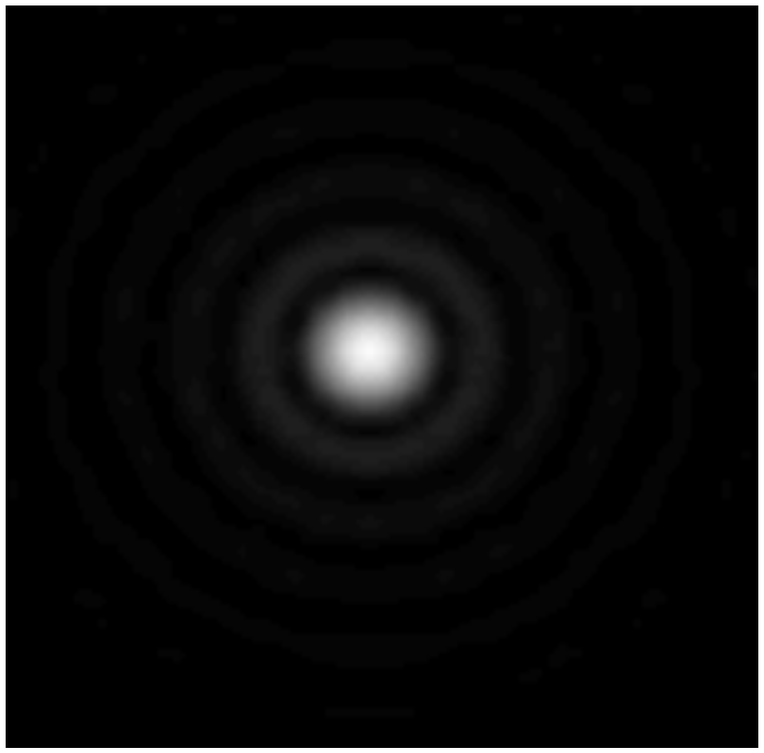


Image plane



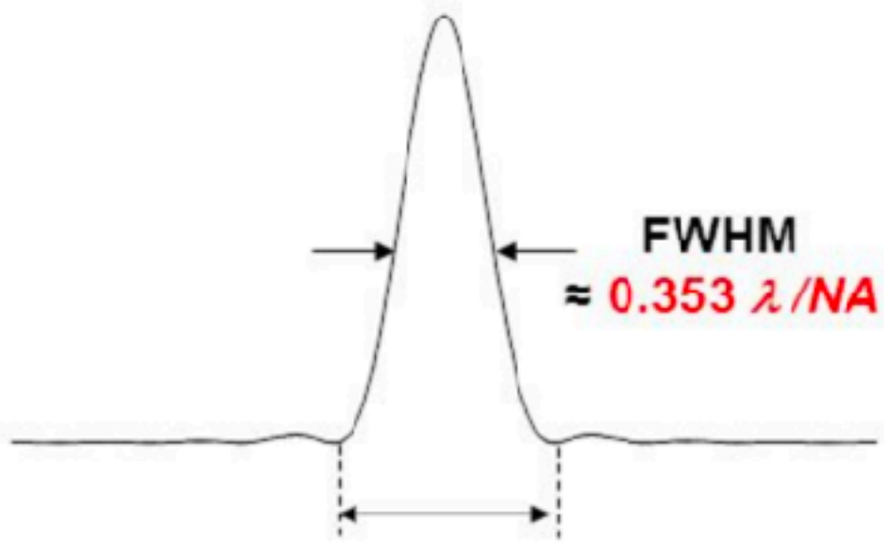
Higher numerical aperture, less distortion

PSF is a way to measure resolution



As the Full Width at Half Max (FWHM) of the PSF

As the diameter of the Airy disk (first dark ring of the PSF)
= “Rayleigh criterion”



PSF of a small object

Image from Jeff Lichtman

OTF (Optical transfer function)

Used in widefield-deconvolution and Super-resolution (SIM)

OTF is the **Fourier transform** of PSF

FT algorithm computes a signal into its **frequency** domain

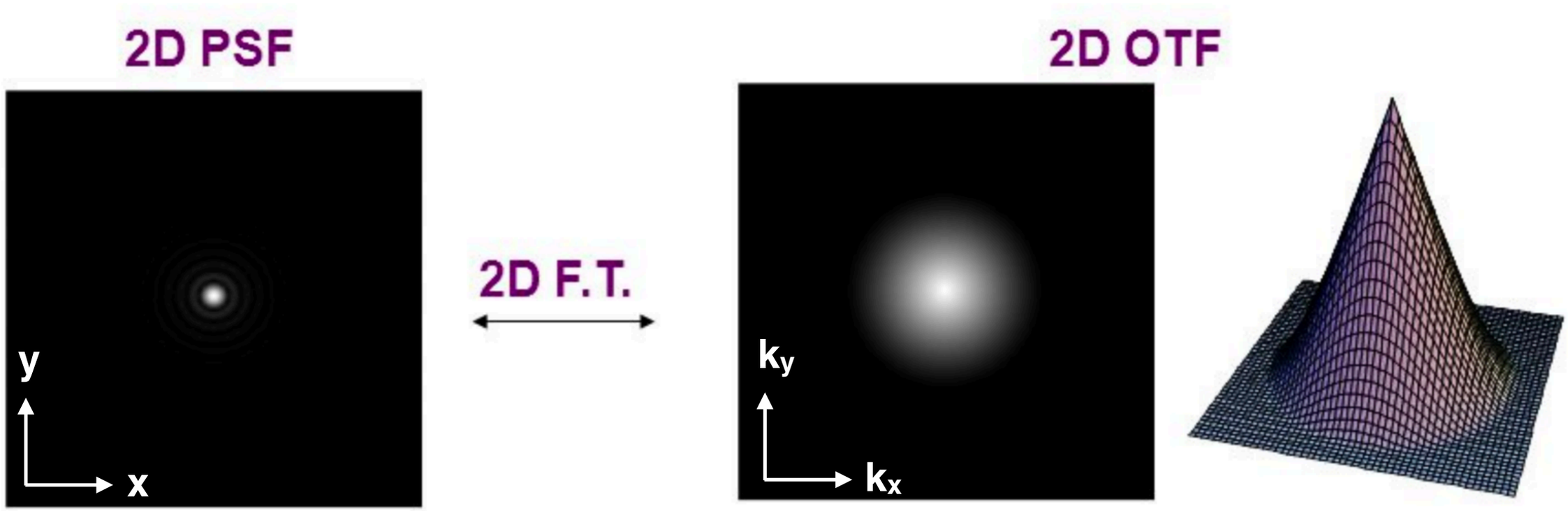
PSF
spatial domain
x, y, z

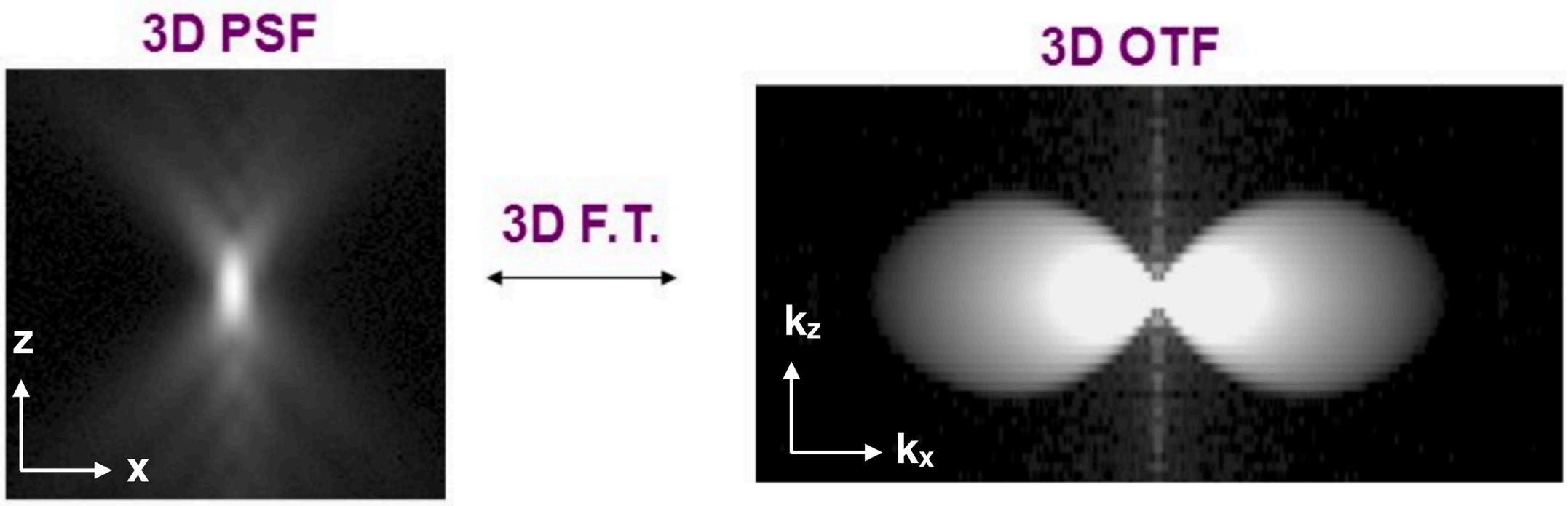
Fourier transform

OTF
spatial frequency domain

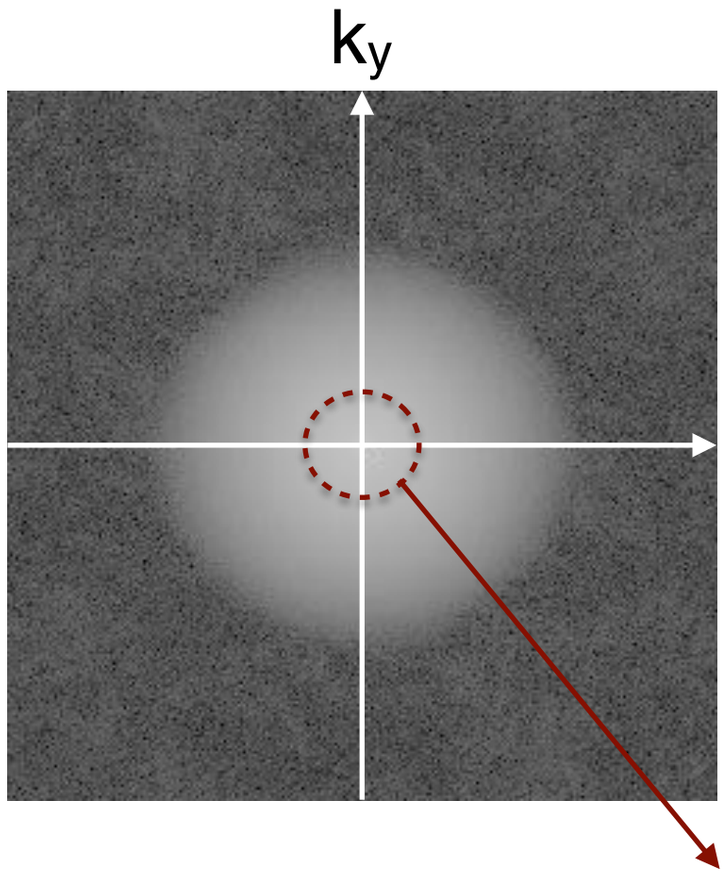
OTF represents how spatial frequencies are handled by the optical system

OTF (Optical transfer function) is the Fourier transform of PSF





OTF (Optical transfer function) is the Fourier transform of PSF

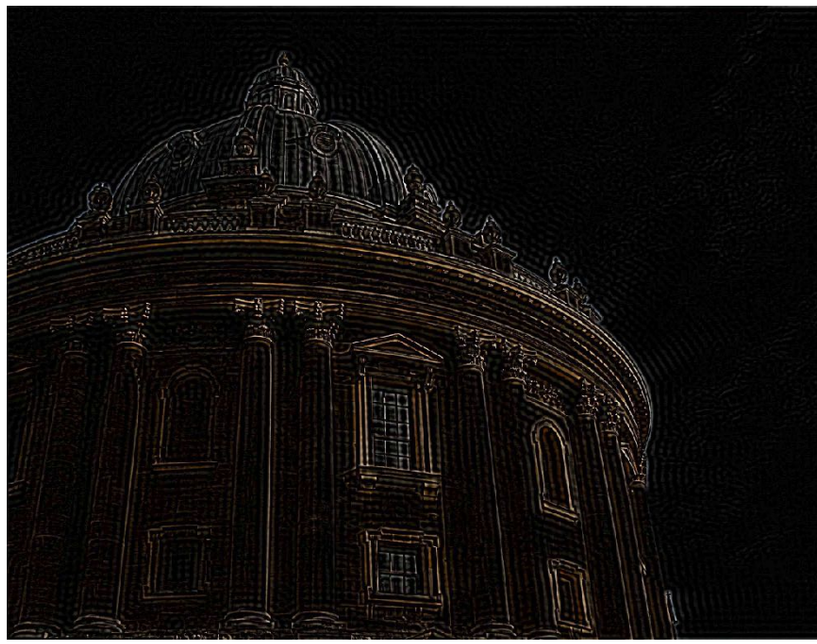


lower frequencies
towards the centre

What are spatial frequencies … in an image?

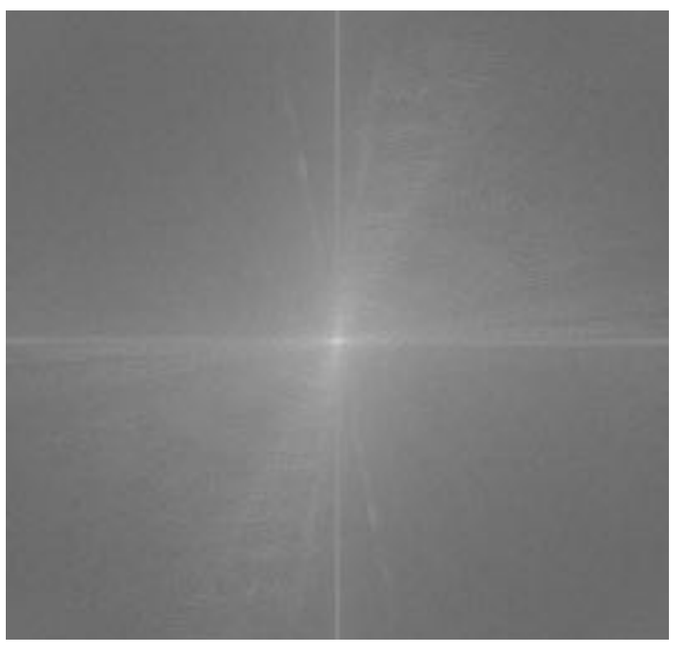
Lower frequencies - blurred

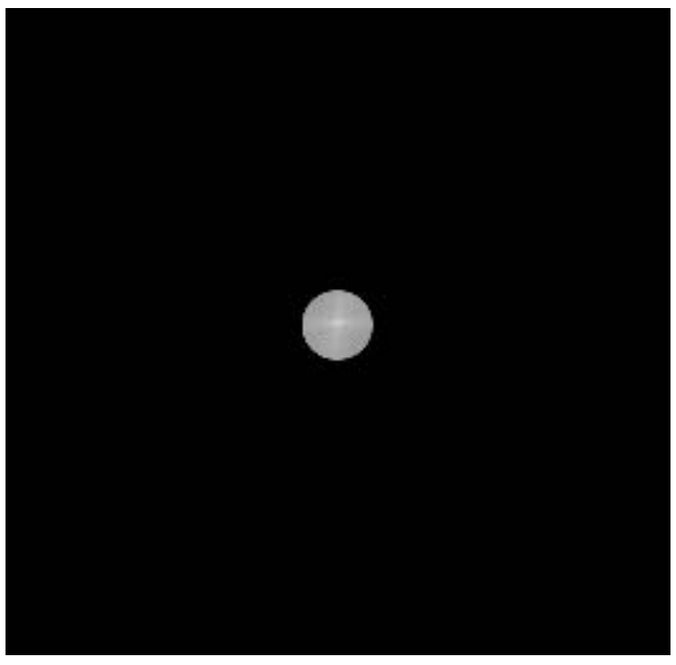
Higher frequencies - sharp

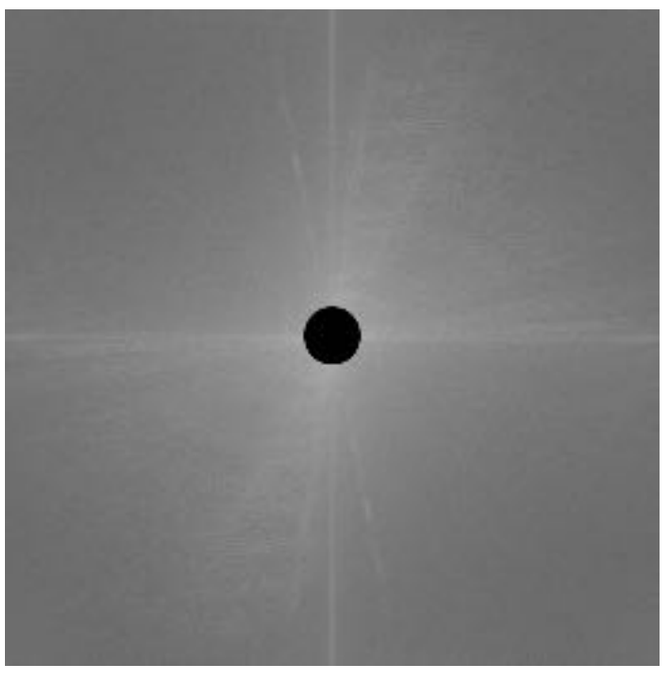


Fourier transform

Inverse Fourier transform

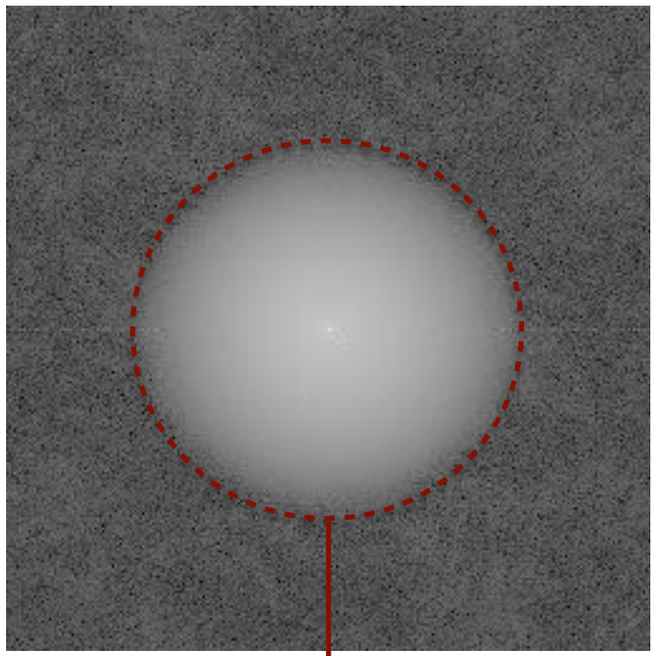




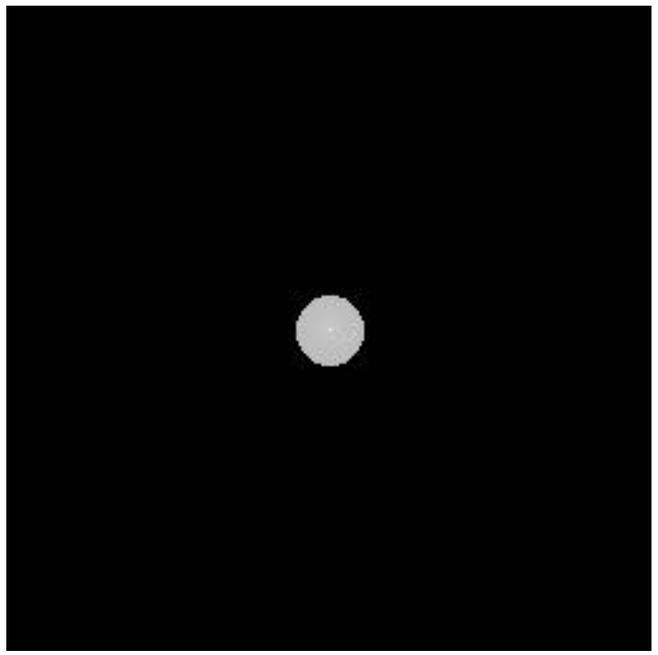


We can very easily discard certain frequencies

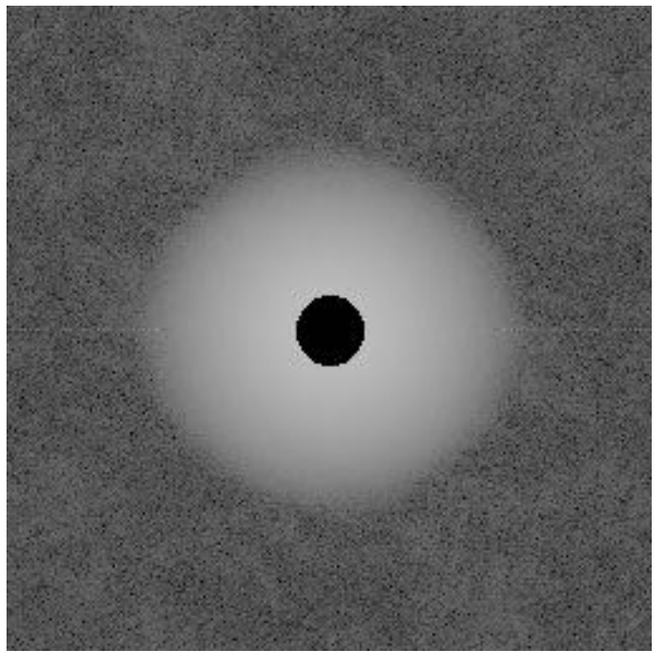
All frequencies



Just lower frequencies



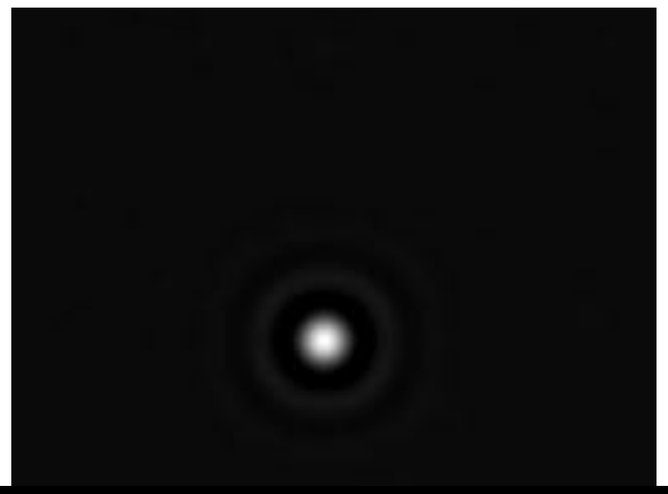
Just higher frequencies

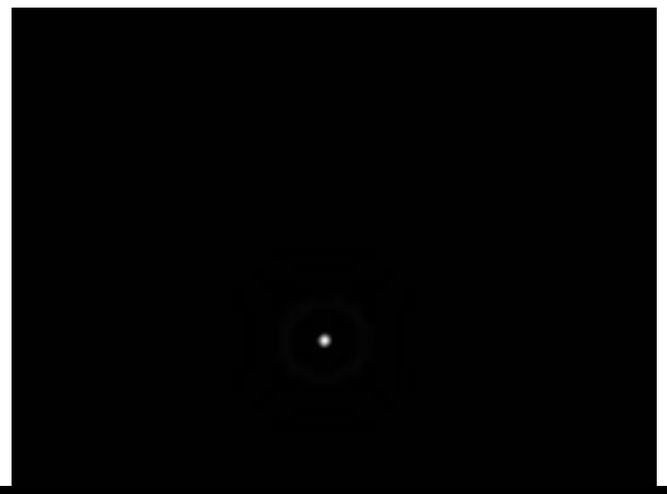


Inverse Fourier transform

What does it represent?

Back Aperture Objective





The microscope passes low frequencies (large and smooth) and excludes high frequencies (greater than 2λ/NA)

Questions?

Technical Tips
(fixed sample preparation)

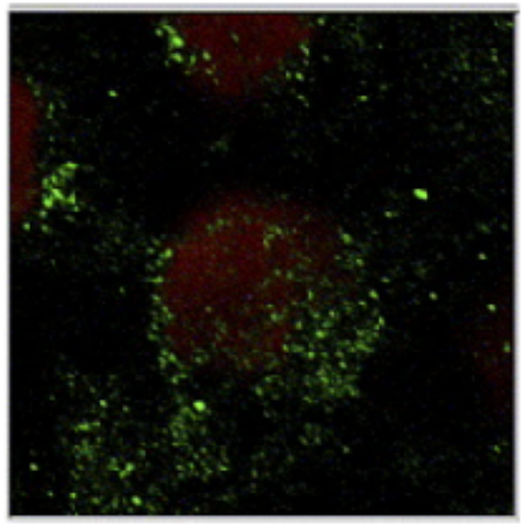
Two Types of Fixation

Denaturing fixation:

Cold methanol or cold acetone stored at -20 °C, samples submerged at -20 °C for 5 to 10 min

- destroys 3D protein structure
- dissolves lipids into micelles
- poor morphological preservation and poor protein retention
- makes some epitopes accessible
- best used after cross-linking fixation

MeOH

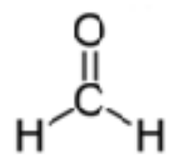


Cross-linking fixation:

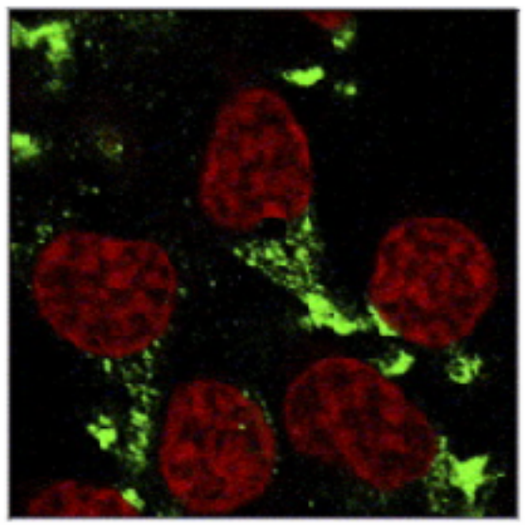
- aldehyde groups cross-link molecules in cells and tissues
- extensive cross-linking prevents antibody penetration

Formaldehyde used for immunocytochemistry in light microscopy

- cross-links 1° amines of Lys and Arg, sulfhydryl groups of Cys, OH groups, double bonds
- binds to amino acids, peptides, proteins and some lipids, but not RNA, DNA or most sugars
- retention of DNA and RNA due to protein cross-linking
- for cultured cells fixation usually for 20 min in 2 - 4% formaldehyde



PFA



Buffers for fixation

- pK range must be 7.0-7.3
- Maintain stable pH and have to have the same tonicity as the cells (same conc. of solutes)
- Usually phosphate buffer but specialist buffers possible: MOPS, TES, HEPES, PIPES

How to prepare cells for fixation

- Grow adherent cells on coverslips for fixation in multiwell plates
- Fix non-adherent cells in suspension after pelleting and resuspending or fix on poly-lysine coated coverslips (0.1mg/mL)

Permeabilisation

Aim: to allow fixative to enter the cells/tissue more quickly if necessary
to allow antibodies to penetrate fixed cells/tissue
done by removing lipids with detergents

Detergents:

- polar lipids with a hydrophilic (water soluble) end and a hydrophobic end that binds the hydrophobic moieties of water insoluble compounds and renders them hydrophilic

Nonionic detergents:

- contain methyl groups that participate in hydrogen bonds and are able to solubilise membranes but do not destroy protein-protein interactions

Triton X-100: used to permeabilise unfixed or lightly fixed eukaryotic cell membranes (0.1% in PBS)
Tween 20: milder than Triton X-100, used to reduce surface tension in blocking, antibody incubation and wash steps (0.1%)
Nonidet P-40 (Igepal Ca-630 from Sigma-Aldrich): used to permeabilise unfixed cells (0.1% in PBS for 5-10s)

Ionic detergents:

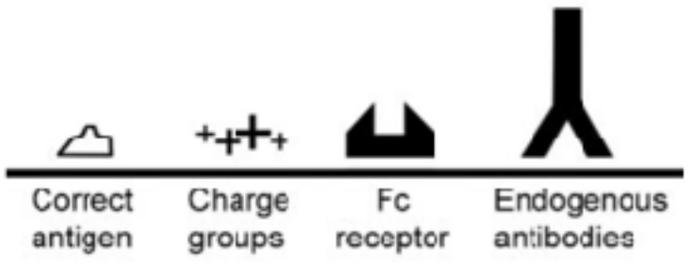
- have highly charged hydrophilic groups and are very effective at solubilising membranes, but also destroy native three dimensional protein structures

SDS, deoxycholate, CHAPS
Not used for immunocytochemistry

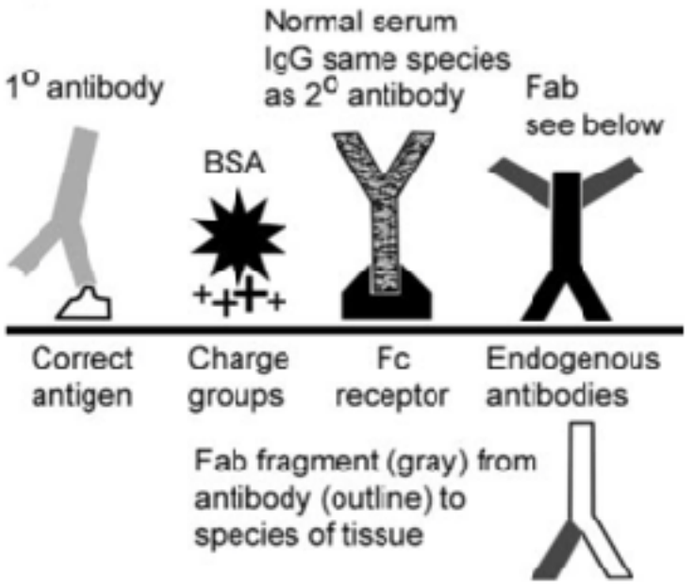
Blocking

Aim: to allow binding of antibodies only to appropriate sites

A



B



Sources of nonspecific binding:

Charged groups

Occur on proteins (esp. histones) or lipids

Also generated by fixation in formalin or glutaraldehyde

To block use bovine serum albumin at 10-30mg/mL (fraction V)

Fc receptors

On macrophages and other immune cells, which bind any antibody

To block whole IgG 1° and 2° antibodies from binding to Fc receptors, incubate cells in buffer containing 5-10% normal serum from the host species of the 2° antibody

Endogenous antibodies

Only a problem for 2° antibodies recognising the same species as your tissue/ cells and only at inflammation sites or in cell cultures of immune system cell types

To block use Fab fragments raised in the same species as the 2° antibody that recognise the species of your tissue/cells as part of the blocking procedure

For general blocking can also try MAXblock (Active Motif): protein based, non-mammalian blocking agent, no cross-reactivity with 2° antibodies

How to choose primary antibodies:

1. Published literature recommendation
2. Product recommended for immunocytochemistry
3. High specificity for the antigen of interest in your species
4. Species the Ab was raised in compatible with other Abs in your experiment

How to store antibodies:

10 µL aliquots in -70 freezer, after defrosting: in fridge for short-term

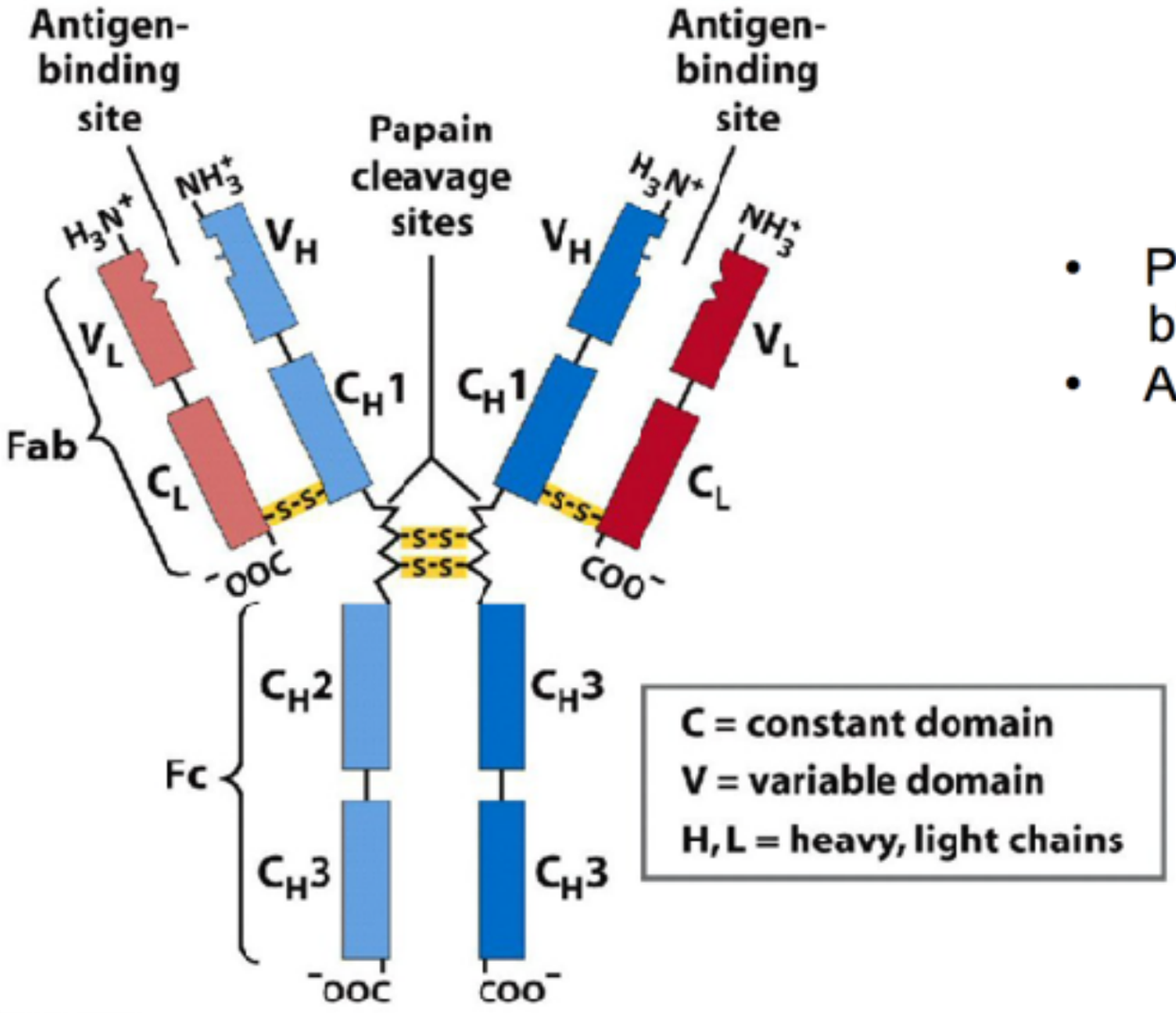


Figure 5-21a
Lehninger Principles of Biochemistry, Fifth Edition

- Preferred: IgG isotype, more consistent generation and binding
- All constant domains are recognised by 2° Abs

Polycolonal antibodies contain multiple clones of antibodies produced to different epitopes of the antigen

Monoclonal antibodies, originally from one mouse, contain a single antibody from one clone of B-cells to a single epitope on the antigen

Affinity-purified Abs best in theory because they have bound to the antigen, but some of the strongest binding Abs cannot be eluted from the affinity columns and are lost.

Polyclonal antibodies

Advantage:

- High levels of labelling because they bind several epitopes on the same protein

Disadvantages:

- Can label multiple proteins that share epitopes
- Different batches have different antibodies

Monoclonal antibodies

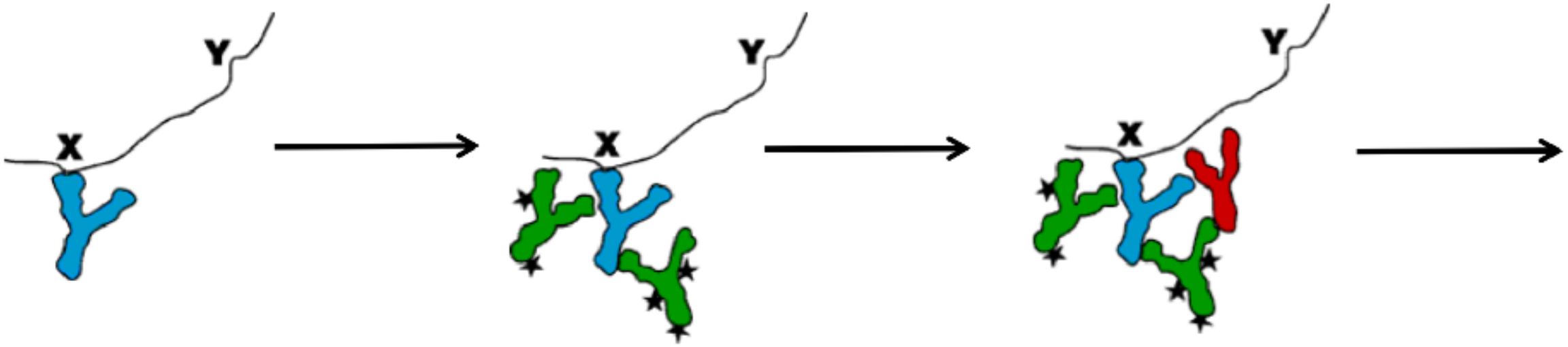
Advantages:

- Single epitope selected for high specificity
- Different clones can be generated to different epitopes on the same antigen
- Single clone can recognise post-transcriptionally modified protein (e.g. phosphorylation)
- Same clone can be generated indefinitely

Disadvantages:

- Low levels of labelling possible
- Mostly from mice

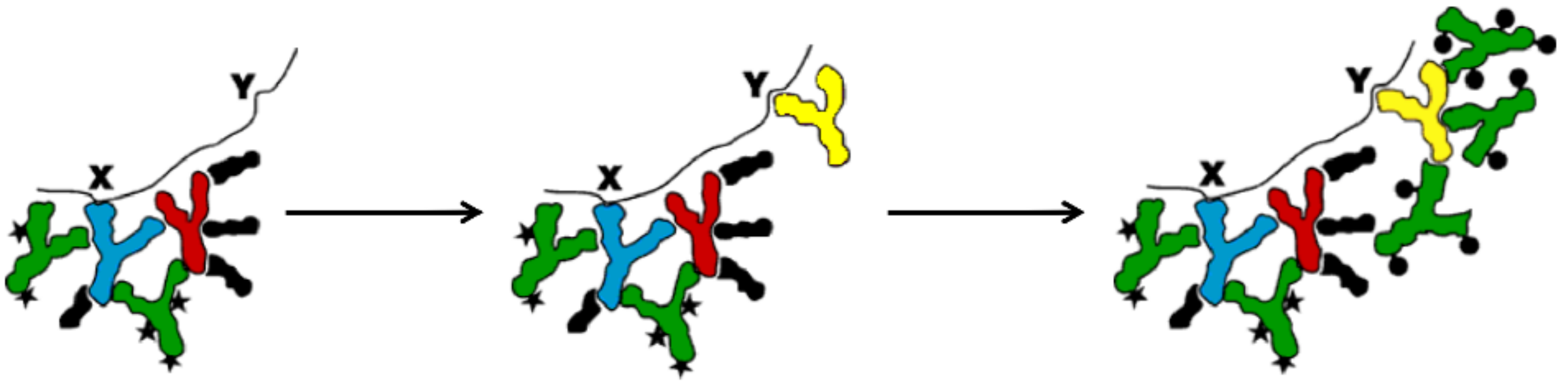
Two 1° antibodies from the same species



rabbit anti-X

goat anti-rabbit IgG Alexa 488

IgG from normal rabbit serum



Fab fragment goat anti-rabbit IgG

rabbit anti-Y

goat anti-rabbit IgG Alexa 568

1° Antibody Controls

- check localisation of fluorescent fusion proteins in live imaging
- compare tissue sections from a normal animal/cells and a knockout animal/cells – not often possible, knockout might not be complete
- single band on western blot or better immunoprecipitation followed by gel and silver staining
- immunocytochemical comparison with known antibody against same target or fluorescent fusion protein

2° Antibody Controls

- omit the 1° antibody and block with normal serum if you see background
- purchase 2° antibodies from reliable manufacturers
- when choosing a 2° antibody for a 1° mouse antibody the 2° frequently needs to be able to bind to the subclass of the IgG used as the 1° antibody

Washes

- Wash with agitation (unless your cells dislodge easily) for 5-10 min for each wash step
- Wash 7 times leaving 10-20% of the buffer each time to prevent drying of your cells/tissue
- Or wash 3 times removing all buffer and replacing it immediately
- If cells/tissue dry out in between washes background is increased and cannot be removed

Washes after the 1° antibody

- Incomplete removal of the 1° antibody does not increase background but lowers the amount o specific labelling because the 2° antibody reacts with the 1° in solution decreasing its conc.

Washes after the 2° antibody

- Incomplete removal of the 2° antibody increases background

Experimental controls: the key for reliable results

Controls for immunofluorescence:

- Autofluorescence: No primary or secondary antibody
- Secondary controls:
  - Incubate with secondary but not primary antibody
  - Prepare samples for each primary antibody individually:
    A. Test cross-talk of the different fluorophores
    B. Test cross-reactivity of secondary antibodies

Experimental controls:

- Compare localisation in live and fixed cells
- Compare antibody reactivity with other known antibodies against the same epitope or target
- Test specificity in knock-out/knock-down cells

Reference Material

http://www.olympusmicro.com/
Very comprehensive and well written

http://micro.magnet.fsu.edu/primer/anatomy/anatomy.html
Very comprehensive

Immunocytochemistry a practical guide for biomedical research
Richard W. Burry, Springer 2010
http://www.jacksonimmuno.com/technical

Molecular Biology of the Cell, fifth edition.
Alberts et al. Chapter 9: Visualizing cells, page 579-616